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Contribution of human esterases to the metabolism of selected new psychoactive substances

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TABLE OF CONTENTS

1 SUMMARY/ ZUSAMMENFASSUNG

1.1 SUMMARY

The aim of this study was to investigate the role of human carboxylesterases (hCES) in the metabolism of selected new psychoactive substances from the class of synthetic cannabinoids. An initial activity screening was conducted incubating synthetic cannabinoids with different structural characteristics with each out of the three most significant hCES isoforms for human drug metabolism, by name hCES1b, hCES1c, and hCES2, to assess their contribution to the synthetic cannabinoids' metabolism. Furthermore, incubations without enzymes were performed as negative controls to elucidate non-enzymatical ester hydrolysis. Kinetic studies were performed for those isoforms, which were shown to significantly contribute to the metabolic transformation. All samples were analyzed using a liquid chromatography-linear ion trap mass spectrometry system.

Results showed that hCES only hydrolyzed the ester bond of synthetic cannabinoids with an amide linker and a terminal ester bond to a significant amount. Furthermore, only SC with a valine-derived structural element, such as MMB-4en-PICA, MMB-FUBINACA, and MMB-CHMICA, and additionally 5F-MPhP-PICA were sufficiently hydrolyzed in order to perform kinetic studies. SC which contained a *tert*-leucine-derived structural element and SC with a terminal amide instead of a terminal ester bond showed only very low hydrolysis rates or no metabolite formation at all. In conclusion, kinetic studies could not be performed for SC of these classes.

In summary, the current study showed that hCES, especially isoforms hCES1b and hCES1c, may play a significant role in the in vivo metabolism of SC with a terminal ester bond and a valine-derived structural element. Additionally, the results of conducted kinetic studies implicate that the molecular weight of the substrate has an impact on its affinity to the respective isoform of hCES1. hCES1c shows higher affinity for larger SC and hCES1b to smaller SC. For instance, in kinetic studies with hCES1b MMB-CHMICA (370 g/mol) showed a higher hydrolysis rate than the heavier substrate 5F-MPhP-PICA (411 g/mol), but further studies are needed to confirm this finding. Finally, hCES-catalyzed hydrolysis of the amide linker may to play a minor role in the in vivo metabolism of SC.

1.2 ZUSAMMENFASSUNG

Das Ziel dieser Studie war es, die Bedeutung von humanen Carboxylesterasen (hCES) für den Metabolismus ausgewählter synthetischer Cannabinoide (SC) mit verschiedenen strukturellen Eigenschaften zu erforschen. Zuerst wurde ein Aktivitätsscreening durchgeführt, bei dem die synthetischen Cannabinoide mit den drei für den menschlichen Fremdstoffmetabolismus wichtigsten hCES Isoformen (hCES1b, hCES1c, hCES2) inkubiert wurden. Enzymkinetiken wurden für die Isoformen erstellt, die eine signifikante Hydrolyseaktivität zeigten. Alle Proben wurden mithilfe eines Ionenfallen-Massenspektrometers, welches an einen Flüssigchromatographen gekoppelt war, analysiert.

Die Ergebnisse zeigten, dass nur die Hydrolyse des Esters bei SC mit Amidlinker und terminaler Estergruppe stark von hCES katalysiert wird. Eine ausreichend starke Hydrolyseaktivität für die Anfertigung von Enzymkinetiken wurde bei 5F-MPhP-PICA und bei SC festgestellt, die ein von der Aminosäure Valin abgeleitetes Strukturmotiv enthielten, so wie es bei MMB-4en-PICA, MMB-FUBINACA und MMB-CHMICA der Fall ist. Im Gegensatz dazu wurden SC mit einem von *tert*-Leucin abgeleiteten Substrukturelement und SC ohne terminale Estergruppe aber mit Amidlinker kaum hydrolysiert, sodass keine Kinetikstudien durchgeführt werden konnten.

Es lässt sich schlussfolgern, dass hCES, vor allem die hCES1-Isoformen, eine signifikante Rolle im in vivo Metabolismus von SC spielen könnte, welche eine terminale Estergruppe und ein von Valin abgeleitetes Strukturmotiv besitzen. Des Weiteren implizieren die durchgeführten kinetischen Studien, dass das Molekulargewicht des Substrates für die Affinität zu der entsprechenden hCES Isoform von Bedeutung sein könnte, da hCES1c eine stärkere Affinität zu schweren SC aufweist, wohingegen hCES1b stärkere Affinitäten zu leichten SC zeigt. Zum Beispiel waren die in Kinetikstudien mit hCES1b ermittelten Hydrolyseraten für MMB-CHMICA (370 g/mol) höher als für 5F-MPhP-PICA (411 g/mol). Um diese Ergebnisse zu bestätigen sollten allerdings weitere Studien durchführt werden. Eine untergeordnete Rolle scheinen hCES in der Hydrolyse des Amidlinkers der SC zu spielen.

2 INTRODUCTION

Human carboxylesterases (hCES) are serine esterases known to play an important role in the catalytic hydrolysis of compounds that contain esters, amides, carbamates, or thioesters [1]. Substrates may be endogenous compounds, but also xenobiotics such as therapeutic agents or drugs of abuse [1]. Investigations on the phase I metabolism of drugs and drugs of abuse demonstrated, that hCES are essential for detoxification processes (e.g. in case of cocaine [2]) but also for the activation of prodrugs (e.g., imidapril [3]). Five hCES subfamilies, hCES1-5, were identified [4], with hCES1 and hCES2 being involved in human drug metabolism. Especially the hCES1 subfamily plays an essential role in the drug metabolism as agents from nearly all therapeutic classes were identified as hCES1 substrates [5]. hCES are localized in the endoplasmatic reticulum of cells in many tissues throughout the human body [1]. While hCES2 is primarily expressed in the gastrointestinal tract and kidneys, the expression of hCES1 is predominantly located in liver and lungs [1, 2]. In the human liver, the main drug metabolizing organ in humans, expression levels of hCES1 are even higher than those of cytochrome P450 as well as UDP-glucuronosyltransferase isoforms [6, 7].Two isoforms of hCES1, by name hCES1b and hCES1c, were identified. They differ in some point mutations and therefore in their substrate specificity with hCES1b being the isoform which is predominant in the human liver [1, 8],

Human carboxylesterases in general are known to be subject to several genetic polymorphism which can lead to interindividual activity differences able to influence the drug metabolism and the clinical outcome of a drug therapy [3, 9, 10]. Furthermore, previous studies showed that several natural products such as cannabis or ginsenosides have an impact on hCES1 activity [7, 11]. For instance, the three major cannabinoids of *Cannabis sativa*, tetrahydrocannabinol (THC), cannabidiol, and cannabinol, were shown to reduce the hepatic hydrolysis of heroin, which is a substrate of the hCES1 subfamily [7, 12]. Moreover, hCES is known to be inhibited by various substances such as flavonoids, naturally occurring fatty acids, or organophosphates [13]. Hence, the role of carboxylesterases in the metabolism of drugs (of abuse) might be considered in order to predict interactions or interpret toxicological findings.

While numerous publications about the involvement of hCES in the metabolism of medications are available, only few studies have been conducted demonstrating that hCES are also involved in the metabolism of drugs of abuse such as cocaine, opiates, and alkaloids [12, 14, 15]. However, only very limited data is available concerning their role in the metabolic fate of new psychoactive substances (NPS) [16, 17]. Yet, NPS play an important role on the drugs of abuse market, with an average of 50 new substance being detected by the EU early warning system annually [18]. The NPS include a variety of different pharmacological groups such as synthetic cannabinoids (SC), phenethylamines, designer benzodiazepines or opioids. This

study focused on SC since some SC, by name AB-PINACA, AB-FUBINACA, BB-22, PB-22 and 5F-PB-22, have already been shown to be hydrolyzed by hCES [16, 17].

SC mimic the pharmacological effects of THC. THC activates CB_1 receptors, which are known to be part of the endocannabinoid system, and affect behavior, mood, percipience of pain [19], and induce the feeling of being 'high' or 'stoned' [20]. Even though the effects of SC are comparable to those of cannabis, severe and fatal poisonings are more common after use of synthetic cannabinoids [21]. Severe side effects after SC consumption include cardiovascular toxicity, rapid loss of consciousness, coma, respiratory depression, and seizures amongst others [22]. The reason for those severe side effects is not yet very well understood, but two factors are likely to play an important role, one of them being the unintentional ingestion of high doses. In the process of mixing SC with plant material in order to produce a smoking mixture, high concentrations of SC in the product are easily obtained [23]. The second reason might be the higher potency of SC compared to THC [18] as SC are full agonists at the CB_1-receptor in contrast to THC [24].

SC are sold as a replacement for cannabis often labeled as 'spice' or 'legal high'. From the mid-2000s until June 2018 179 different SC were identified in the EU [25]. In 2018, SC posed the second largest group of NPS seized in the EU, directly behind synthetic cathinones as largest group [25]. The general structure of SC is shown in Figure 1, consisting of a tail, a core structure and a linked group (= secondary moiety) which is coupled to the core by a linker [26]. In order to study the metabolism of a wide range of SC we included different groups of SC which all contain an amide linker and some of which also show a terminal amide or ester bond at the secondary moiety (see Figure 2). Group 1 consisted of nine SC containing an amide linker and a terminal methyl ester bond at the secondary moiety. 5F-MDMB-PICA, MDMB-FUBICA, 4F-MDMB-BINACA, and MDMB-4en-PINACA formed a subgroup with a *tert*-leucine-derived structural motif in their linked group. Another subgroup is formed by MMB-4en-PICA, MMB-FUBINACA, MMB-CHMICA, and 5F-MMB-PICA with a valine-derived structural motif in their linked group. 5F-MPhP-PICA, which is also part of group 1, has a benzyl group in its secondary moiety. MBA-CHMINACA and DMBA-CHMINACA formed group 2 with an amide linker and terminal carboxylic acid. Group 3 consisted of 3,5-5F-AB-FUPPYCA, 5F-AB-P7AICA, and AB-PINACA containing an amide linker and a terminal amide at the secondary moiety. The fourth group only consisted of A-CHMINACA which only has an amide linker but no terminal amide, carboxylic acid, or ester bond at the secondary moiety.

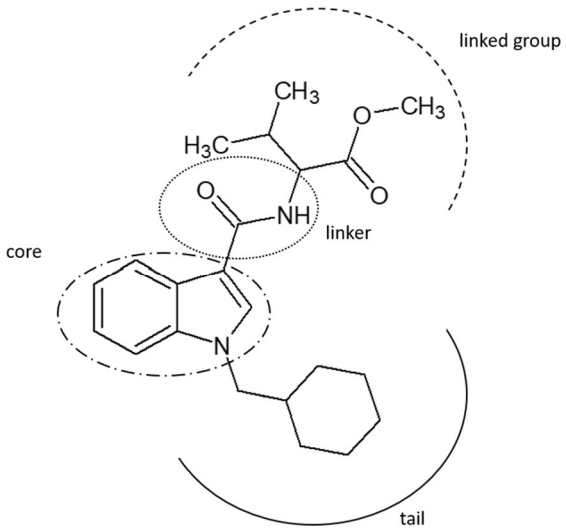

Figure 1 *Structural scheme of synthetic cannabinoids consisting of core, linker, linked group, and tail*

Hence, in order to obtain an improved understanding of the role of hCES in SC metabolism and therefore to be able to better predict interactions or interpret toxicological findings of these different groups of SC we conducted initial activity screenings using incubations with hCES, pooled human liver microsomes (pHLM), and pooled human liver S9 fraction (pHLS9) followed by an analysis with liquid chromatography-ion trap mass spectrometry (LC-ITMS). Furthermore, Michaelis-Menten kinetic studies were performed using incubations with hCES.

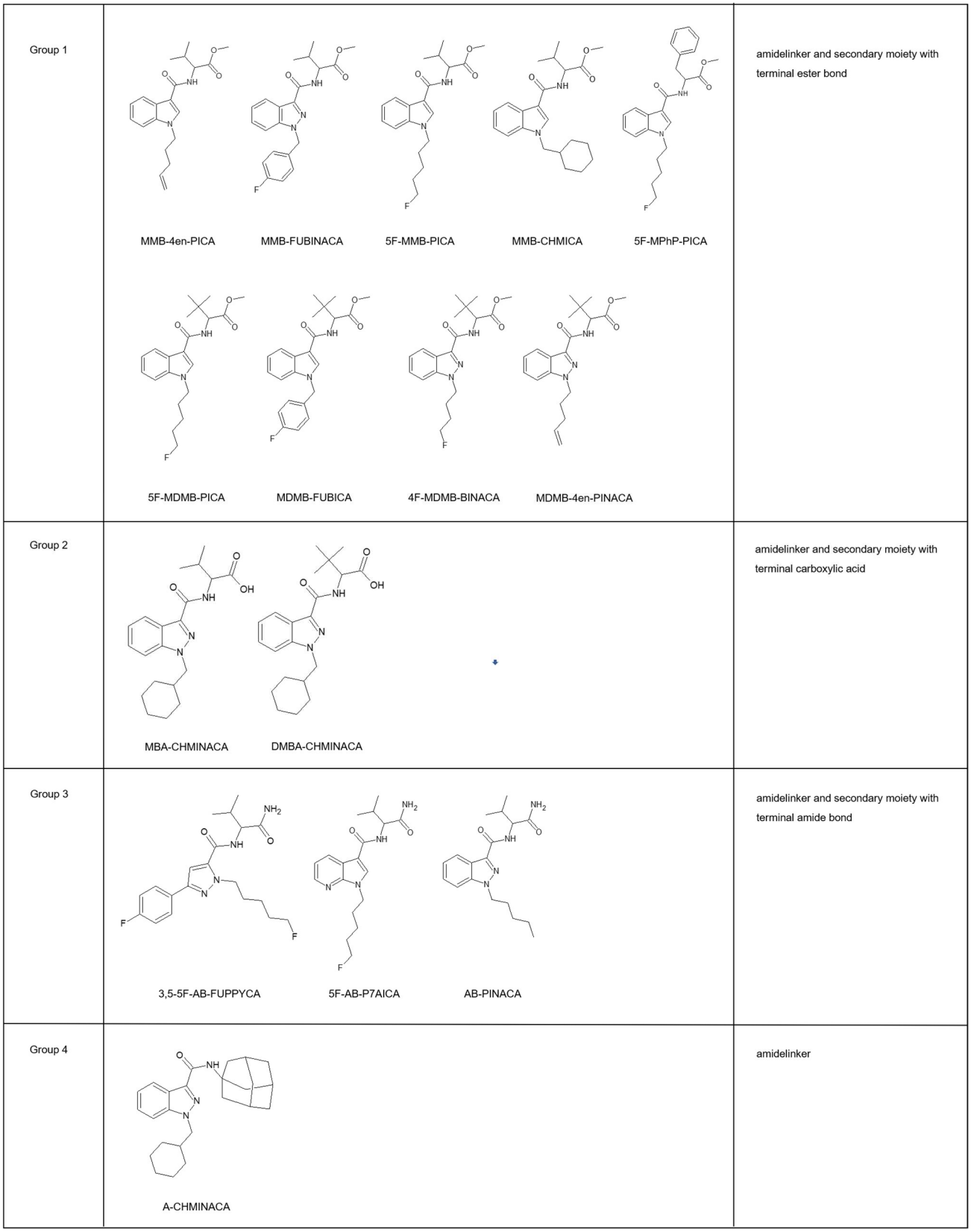

Figure 2 *Chemical structures of tested synthetic cannabinoids divided into groups 1-4 according to their structural characteristics*

3 MATERIAL AND METHODS

3.1 REAGENTS AND MATERIALS

MDMB-FUBICA, 5F-MDMB-PICA, DMBA-CHMINACA, MBA-CHMINACA, A-CHMINACA, 5F-AB-P7AICA, MMB-4en-PICA, MDMB-4en-PINACA, 5F-MPhP-PICA, 4F-MDMB-BINACA, 3,5-5F-AB-FUPPYCA, and MMB-CHMICA were provided by the EU-funded project ADEBAR (IZ25-5793-2016-27). MMB-FUBINACA and 5F-MMB-PICA were provided by the Institute of Forensic Medicine, University of Freiburg (Freiburg, Germany). AB-PINACA was obtained from Cayman Chemicals (Michigan, USA). Thebacon was available from a previous study and kindly provided by Prof. Robert Ammon (Homburg, Germany). Trimipramine-D3 was obtained from LGC standards (Wesel, Germany).

Recombinant hCES1b, hCES1c, and hCES2 (prepared from baculovirus transfected insect cells) as well as pHLM (20 mg microsomal protein/mL) and pHLS9 (20 mg protein/mL) were from Corning (Amsterdam, The Netherlands). After delivery, the enzyme containing preparations were thawed at 37°C, aliquoted, snap-frozen in liquid nitrogen, and stored at -80°C until use.

Potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), and dimethyl sulfoxide (DMSO) were obtained from Merck KGaA (Darmstadt, Germany). All other solvents were purchased by VWR (Darmstadt, Germany).

Methanolic stock solutions of 5F-MDMB-PICA, MDMB-FUBICA, 4F-MDMB-BINACA, MDMB-4en-PINACA, MMB-4en-PICA, 5F-MPhP-PICA, MBA-CHMICA, DMBA-CHMICA, 3,5-5F-AB-FUPPYCA, 5F-AB-P7AICA, AB-PINACA and A-CHMINACA (1 mg/mL), MMB-FUBINACA (2 mg/mL), AB-PINACA (3 mM) and DMSO stock solutions of MMB-CHMICA and 5F-MMB-PICA (50mM) were used for the initial screening experiments. DMSO stock solutions of Thebacon (100 mM), MMB-4en-PICA (50 mM), 5F-MPhP-PICA (50 mM), MMB-CHMICA (50 mM), and MMB-FUBINACA (100 mM) were used for the enzyme kinetics.

3.2 INITIAL ACTIVITY SCREENING WITH HUMAN CARBOXYLESTERASES (HCES1B, HCES1C AND HCES2), PHLM, AND PHLS9

Initial activity screenings were carried out as previously described by Meyer *et al.* [12] with the test compound thebacon as well as the SC 5F-MDMB-PICA, MDMB-FUBICA, 4F-MDMB-BINACA, MDMB-4en-PINACA, MMB-4en-PICA, MMB-FUBINACA, MMB-CHMICA, 5F-MMB-PICA, 5F-MPhP-PICA, MBA-CHMICA, DMBA-CHMICA, 3,5-5F-AB-FUPPYCA, 5F-AB-P7AICA, AB-PINACA, A-CHMINACA. One of the substrates (final concentration, 100 µM) was incubated at 37°C for 30 minutes with hCES1b, hCES1c, or hCES2 (final protein concentration,

0.2 µg/µL, each) or pHLM or pHLS9 (final protein concentration, 2 µg/µL, each). The final volume of the incubation mixture was 100 µL, consisting of substrate, enzyme-containing preparation, and phosphate buffer (100 mM, pH 7.4). Negative controls without enzyme were also prepared to monitor non-enzymatic hydrolysis. All incubations were done in duplicate. Organic solvent concentration in all incubation was below 4%. The reactions were started by adding the enzyme-containing preparation and stopped by adding an equal volume of ice-cold acetonitrile containing 1 µM internal standard trimipramine-D3. All samples were centrifuged at 18,407×g for 15 minutes, 50 µL of the supernatant phase were transferred to autosampler vials, and 10 µL injected onto the LC-ITMS apparatus for analysis.

3.3 Initial activity screening with test compound AB-PINACA

The initial activity screening with AB-PINACA was conducted as previously described by Thomsen *et al.* [16] with minor modifications. AB-PINACA (final concentration, 10 µM) was incubated at 37°C for 20 minutes with hCES1b, hCES1c, or hCES2 (final protein concentration, 0.1 µg/µL, each) or pHLM or pHLS9 (final protein concentration, 1 µg/µL, each). The final volume of the incubation mixture was 100 µL, consisting of substrate, enzyme-containing preparation, and phosphate buffer (100 mM, pH 7.4). Negative controls without enzyme were also prepared to monitor non-enzymatic hydrolysis. All incubations were done in duplicate. After 0, 10, and, 20 minutes sampling was performed by transferring aliquots of 20 µL to an equal volume of ice-cold acetonitrile, containing 0.5% formic acid and 0.01 µM internal standard trimipramine-D3. Afterwards, 110 µL of 0.1% formic acid in water was added. All samples were centrifuged at 2000×g, 5°C, for 10 minutes, 50 µL of the supernatant phase were transferred to autosampler vials and 1 µL injected into the LC-HRMS/MS apparatus for analysis.

3.4 Enzyme kinetic studies

Enzyme kinetic studies were done for the test compound thebacon and the SC MMB-4en-PICA, MMB-FUBINACA, MMB-CHMICA, and 5F-MPhP-PICA with the hCES isoform which showed sufficient activity in the initial activity screening. Thebacon was used as test compound for hCES1b, hCES1c and hCES2, MMB-4en-PICA was incubated with hCES1c, 5F-MPhP-PICA with hCES1b and hCES1c, MMB-CHMICA with hCES1b, and MMB-FUBINACA with hCES1b and hCES1c.

Kinetic studies were conducted using the conditions given in Table 1. In order to determine kinetic parameters (K_m, V_{max}) enzyme concentration and incubation time were chosen to be

within linear range of metabolite formation. For determination, substrate (final concentration, 100 µM) was incubated with different protein concentrations (final concentrations, 0.0125, 0.025, 0.05, 0.1, and 0.2 µg/µL) for 15 minutes at 37°C. All other incubation conditions were comparable to those of the initial activity screening. An enzyme concentration in the linear range of metabolite formation was chosen for determination of incubation time for further kinetic studies. In order to determine the incubation time for the kinetic studies, substrate (final concentration, 100 µM) was incubated for 1, 2, 5, 10, 15, 20, 25, and 30 minutes at 37°C with the previously determined final protein concentration. For each incubation time a negative control was also incubated to monitor non-enzymatic hydrolysis. An incubation time in the linear range of metabolite formation was chosen for kinetic studies.

Table 1 *Calculated kinetic constants to determine enzyme kinetics of the 5 studied compounds*

Substrate	Enzyme	Incubation Time (min)	Final Protein Concentration (µg/µL)	Final Substrate Concentrations, µM
Thebacon	hCES1b	5	0.05	0-3000
	hCES1c	5	0.05	0-2000
	hCES2	5	0.05	0-2000
MMB-4en-PICA	hCES1c	30	0.1	0-2000
MMB-FUBINACA	hCES1b	15	0.05	0-500
	hCES1c	10	0.05	0-1000
MMB-CHMICA	hCES1b	15	0.05	0-250
5F-MPhP-PICA	hCES1b	20	0.05	0-500
	hCES1c	5	0.1	0-250

Incubation mixtures (final volume, 100 µL) for the kinetic studies consisted of substrate, enzyme, and phosphate buffer. In all incubations DMSO concentration was at or below 2%, except for studies with thebacon (hCES1b, 6%, hCES1c and hCES2, 4%) and MMB-4en-PICA (hCES1c, 6%). Negative controls without enzyme were also prepared to monitor non-enzymatic hydrolysis. All incubations were done in duplicate. The reactions were started by adding the enzyme and stopped by adding an equal volume of ice-cold acetonitrile containing 1 µM internal standard trimipramine-D3. The solutions were centrifuged at 18,407×g for 15 minutes, 50 µL of the supernatant phase was transferred to autosampler vials, and 10 µL injected onto the LC-ITMS apparatus for analysis.

3.5 Verification of Spontaneous Ester Hydrolysis

3.5.1 Incubation time dependency

MMB-4en-PICA (100 µM, 500 µM, 1000 µM, 2000 µM, and 3000 µM) was incubated in phosphate buffer at 37°C for 20 minutes without any enzyme containing system. The final volume of the incubation mixture was 250 µL. Sampling took place after 0 and 20 minutes by transferring aliquots of 100 µL to an equal volume of ice-cold acetonitrile containing 1 µM internal standard trimipramine-d3. All samples were centrifuged at 18,407×g for 15 minutes, 50 µL of the supernatant phase was transferred to autosampler vials, and 10 µL injected onto the LC-ITMS apparatus for analysis.

3.5.2 Aqueous milieu dependency

MMB-4en-PICA was diluted to 100 µM, 500 µM, 1500µM, and 3000 µM in phosphate buffer and in acetonitrile. Samples of 100 µL were immediately transferred to an equal volume of ice-cold acetonitrile containing 1 µM internal standard trimipramine-d3. All samples were then centrifuged at 18,407×g for 15 minutes, 50 µL of the supernatant phase was transferred to autosampler vials, and 10 µL injected onto the LC-ITMS apparatus for analysis.

3.6 LC-MS/MS Apparatus

According to previous studies [12, 27] analytes were separated by a TF Accela ultra-high performance liquid chromatography (UHPLC) system consisting of a degasser, a quaternary pump and an autosampler. The LC-system was coupled to a Thermo Fisher Scientific LXQ linear ion trap mass spectrometer (TF, Dreieich, Germany) equipped with a heated electrospray ionization (HESI) II source which was used for analysis. The following LC conditions were chosen: TF Hypersil GOLD C18 column (100 x 2.1 mm, 1.9 µm) guarded by a TF Hypersil GOLD C18 drop-in guard cartridge and a TF Javelin column filter. Gradient elution was performed with 10 mM aqueous ammonium formate buffer containing 0.1% (v/v) formic acid as mobile phase A and acetonitrile containing 0.1% (v/v) formic acid as mobile phase B. The flow rate was set to 500 µL/min and the gradient was programmed as follows: 0-1.0 min 98% A, 1.0-8.0 min to 30% A, 8.0-8.01 to 0% A and 8.01-10 min hold 0% A. Summing up, the gradient had a total run time of 10 minutes with start conditions of 98% A to final conditions of 0% A at 500 µL/min. Re-equilibration of the chromatographic system was performed in a separate run.

The MS conditions were in accordance with a previous study [12]. Briefly, data were acquired

by using the positive electrospray ionization mode. Further MS parameters were sheath gas, nitrogen at flow rate of 34 arbitrary units (AU); auxiliary gas, nitrogen at flow rate of 11 AU; vaporizer temperature, 250°C; source voltage, 3.00 kV; ion transfer capillary temperature, 300°C; capillary voltage, 31 V; and tube lens voltage, 80 V. Automatic gain control was set to 15,000 ions for full scan and 5,000 ions for MS^n. For full scan analysis (MS^1 stage), the maximum injection time was set to 100 ms. Precursor ions were selected from MS^1 using information-dependent acquisition (IDA) with subsequent collision-induced dissociation-MS^n experiments. Hence, MS^1 was performed in the full scan mode (*m/z* 100-800) and MS^2 and MS^3 were performed in IDA mode. The following settings were used for IDA mode: four IDA MS^2 scan filters were chosen to provide MS^2 on the four most intense signals from MS^1, eight MS^3 scan filters were chosen to record MS^3 on the two most intensive signals from MS^2. Furthermore, MS^2 spectra were collected with higher priority than MS^3 spectra. Normalized wideband collision energies were set to 35.0% for MS^2 and 40.0% for MS^3. Further settings for MS^2 were as follows: minimum signal threshold, 100 counts; isolation width, 1.5 u. Further settings for MS^3 were as follows: minimum signal threshold, 50 counts; isolation width, 2.0 u. Further settings for both stages were: activation Q, 0.25; activation time, 30 ms. The dynamic exclusion mode was set as follows: repeat counts, 2; repeat duration, 15 s; exclusion list, 50; exclusion duration, 15 s, and average full scan to full scan cycle time, 4 s. For data handling the Xcalibur Qual Browser software version 4.0 (TF, Dreieich, Germany) was used.

Metabolites formed during the initial activity screening were identified due to the fragmentation pattern in their ITMS spectra in comparison to the ITMS spectra of the parent compounds. While using full scan mode with IDA was used for analysis of the initial activity screening samples, kinetic study samples were measured using a full MS^2 product ion spectra (PIS) of predefined protonated molecules of all target analytes, hydrolysis products, and the internal standard. Monitored masses of the target analytes and hydrolysis products were *m/z* 342 and 300 for thebacon, *m/z* 343 and 329 for MMB-en-PICA, *m/z* 411 and 397 for 5F-MPhP-PICA, *m/z* 384 and 370 for MMB-FUBINACA, and *m/z* 371 and 357 for MMB-CHMICA. The monitored mass of the internal standard trimipramine-D3 was *m/z* 103.

3.7 LC-HRMS/MS APPARATUS

To analyze the samples of the initial activity screening of AB-PINACA a Thermo Fisher Scientific (TF, Dreieich, Germany) Dionex UltiMate 3000 RS pump equipped with a degasser, a quaternary pump, and an UltiMate RS autosampler coupled to a TF Q-Exactive Plus system with a HESI II source was used as already described by Wagmann *et al.* [28] with some modifications. As recommend by the manufacturer external calibrations were done prior to analysis. The injection volume for all samples was 1 µL. The gradient elution was performed on a TF Accucore PhenylHexyl column (100 mm × 2.1mm, 2.6 µm). Mobile phase A consisted of 2 mM aqueous ammonium formate with 0.1% formic acid (v/v, pH 3) and mobile phase B consisted of 2 mM ammonium formate solution with acetonitrile, containing methanol (1:1, v/v), 1% water (v/v), and 0,1% formic acid (v/v). The gradient was programmed as follows: 0–1 min hold 99% A, 1–10 min to 1% A, 10–11.5 min hold 1% A and 11.5–13.5 min hold 99% A. The flow rate was set to 500 µL/min for the first 10 minutes and to 800 µL/min for minutes 10-13.5. The HESI II source conditions were as follows: heater temperature, 320°C; ion-transfer capillary temperature, 320°C; spray voltage, 4.0 kV; ionization mode, positive; sheath gas, 60 arbitrary units (AU); auxiliary gas, 10 AU; sweep gas, 0 AU and S-lens RF level, 50.0.

Mass spectrometry experiments were carried out in full-scan mode with subsequent data-dependent MS^2 (dd MS^2) with priority set to mass-to-charge ratios (*m/z*) of parent compound AB-PINACA (*m/z* 332.2128), the two possible metabolites forming from hydrolysis (*m/z* 332.1969 and *m/z* 233.1284) and the internal standard trimipramine-D3 (*m/z* 298.2357). Full-scan data acquisition was performed with the following settings: resolution, 35,000; microscans, 1; automatic gain control (AGC) target, 1e6; maximum injection time (IT), 120 ms and scan range, *m/z* 130-930 due to the *m/z* of expected metabolites. For dd MS^2 mode an inclusion list was prepared with the previously mentioned mass-to-charge ratios of AB-PINACA, its expected metabolites, and the internal standard. Furthermore, the following settings were selected: option ‘pick others’, enabled; dynamic exclusion, 2 s; resolution, 17,500; microscans, 1; isolation window, 1.0 *m/z*; loop count, 5; AGC target, 2e5; maximum IT, 250 ms; high-collision dissociation cell with stepped normalized collision energy, 17.5, 35.0, 52.5; exclude isotopes, on and spectrum data type, profile. ChemSketch 2010 12.01 (ACD/Labs, Toronto, Canada) was used for drawing of chemical structures and calculation of their exact mass. For data handling the TF Xcalibur Qual Browser software version 4.0 (TF, Dreieich, Germany) was used.

3.8 Data Analysis

Kinetic parameters were determined by using simple peak areas of the metabolites, which were divided by the corresponding peak area of the internal standard. Metabolic formation was corrected for spontaneous hydrolysis, such as non-enzymatic hydrolysis, by subtracting any metabolite detected in negative control incubations (peak area metabolite/ corresponding peak area IS – peak area metabolite negative control/ corresponding peak area IS).

For initial activity screenings the simple peak area of the formed metabolite of incubations with hCES was divided by the substrate peak area of the negative control and then multiplied by 100 in order to obtain the percentage of substrate which was hydrolyzed by the respective hCES.

Enzyme kinetic constants were determined with non-linear curve-fitting by using GraphPad Prism 5.00 software (San Diego, CA). To calculate K_m (Michaelis-Menten constant) and V_{max} (maximum velocity) values for single-enzyme systems the Michaelis-Menten equation (see Eq. (1)) was used.

$$V = \frac{V_{\max} \times [S]}{K_m + [S]} \qquad \text{Eq. (1)}$$

4 RESULTS

4.1 INITIAL ACTIVITY SCREENING STUDIES

4.1.1 Test compound thebacon

The initial activity screening showed that the test compound thebacon was hydrolyzed by all three isoforms. As shown in Figure 3, the relative amount of the formed metabolite codeine was 47% for hCES1b, 10% for hCES1c, and 19% for hCES2. In incubations with pHLM and pHLS9 the relative amount of formed codeine was higher than 80%.

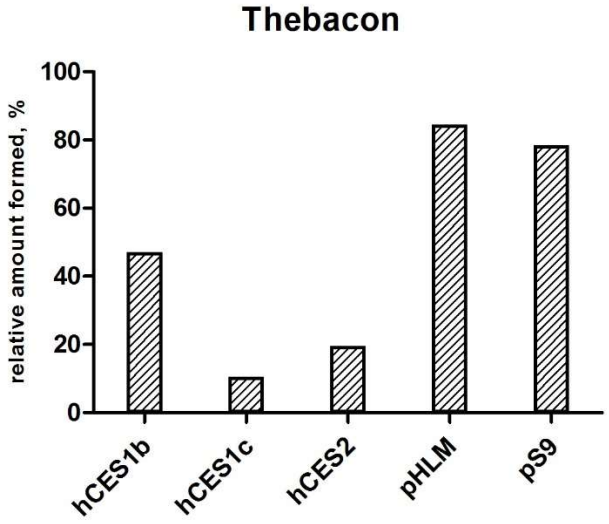

Figure 3 *Initial activity screening studies with the test compound thebacon*

4.1.2 Initial activity screening of selected synthetic cannabinoids

In addition to the test compound thebacon, 15 SC were screened, which were divided into 4 groups according to their chemical structures as previously described. An overview of the results is shown in table 2.

Table 2 *Results of initial activity screenings, + = detected, - = not detected*

	hCES1b	hCES1c	hCES2	pHLM	pHS9
Thebacon	+	+	+	+	+
MMB-4en-PICA	+	+	+	+	+
MMB-FUBINACA	+	+	+	+	+
5F-MMB-PICA	-	-	-	-	-
MMB-CHMICA	+	+	-	+	+
5F-MPhP-PICA	+	+	+	+	+
5F-MDMB-PICA	-	-	-	-	-
MDMB-FUBICA	-	+	-	+	+
4F-MDMB-BINACA	+	+	+	-	+
MDMB-4en-PINACA	+	+	-	+	+
MBA-CHMINACA	-	-	-	-	-
DMBA-CHMINACA	-	-	-	-	-
3,5-5F-AB-FUPPYCA	-	-	-	-	-
5F-AB-P7AICA	-	-	-	-	-
AB-PINACA *	+	-	-	+	+
A-CHMINACA	-	-	-	-	-

hCES which showed highest hydrolysis rate

* initial activity screening with AB-PINACA was perfomed with different experimental conditions and a different analysis method

4.1.2.1 Group 1: amide linker and a terminal methyl ester bond

Group 1 consisted of MMB-4en-PICA, MMB-FUBINACA, MMB-CHMICA, 5F-MMB-PICA, 5F-MPhP-PICA, 5F-MDMB-PICA, MDMB-FUBICA, 4F-MDMB-BINACA, and MDMB-4en-PINACA which all showed an amide linker and a terminal methyl ester bond at the secondary moiety. All metabolites found in the initial activity screening were formed through hydrolysis of the terminal ester bond.

As shown in Figure 4, MMB-FUBINACA showed the highest hydrolysis rates with a relative amount of formed metabolite of 78% for hCES1b and 87% for hCES1c. Furthermore, MMB-FUBINACA was completely hydrolyzed in pHLM and pHLS9.

In case of 5F-MPhP-PICA, hCES1b and hCES1c were identified as the isoforms with the highest hydrolysis rates as well, but in contrast to MMB-FUBINACA, the relative amount of formed metabolite was only around 20% and the hydrolysis rate of pHLM and pHLS9 wasn't higher than 21% and 20%, respectively.

MMB-CHMICA appeared to be mainly hydrolyzed by hCES1b with a hydrolysis rate of 14%. Here the hydrolysis rate of hCES1c was much lower with only 2.3% and the hydrolysis rate of pHLM and pHLS9 was slightly higher with 25% for pHLM and 20% for pHLS9.

In the initial activity screening with MMB-4en-PICA, hCES1c was shown to be the most active isoform with a hydrolysis rate of 5.7%. For pHLM and pHLS9 the relative amount of metabolite formed was 16% (pHLM) and 11% (pHLS9). The initial activity screening of 5F-MMB-PICA did not show any metabolite formation.

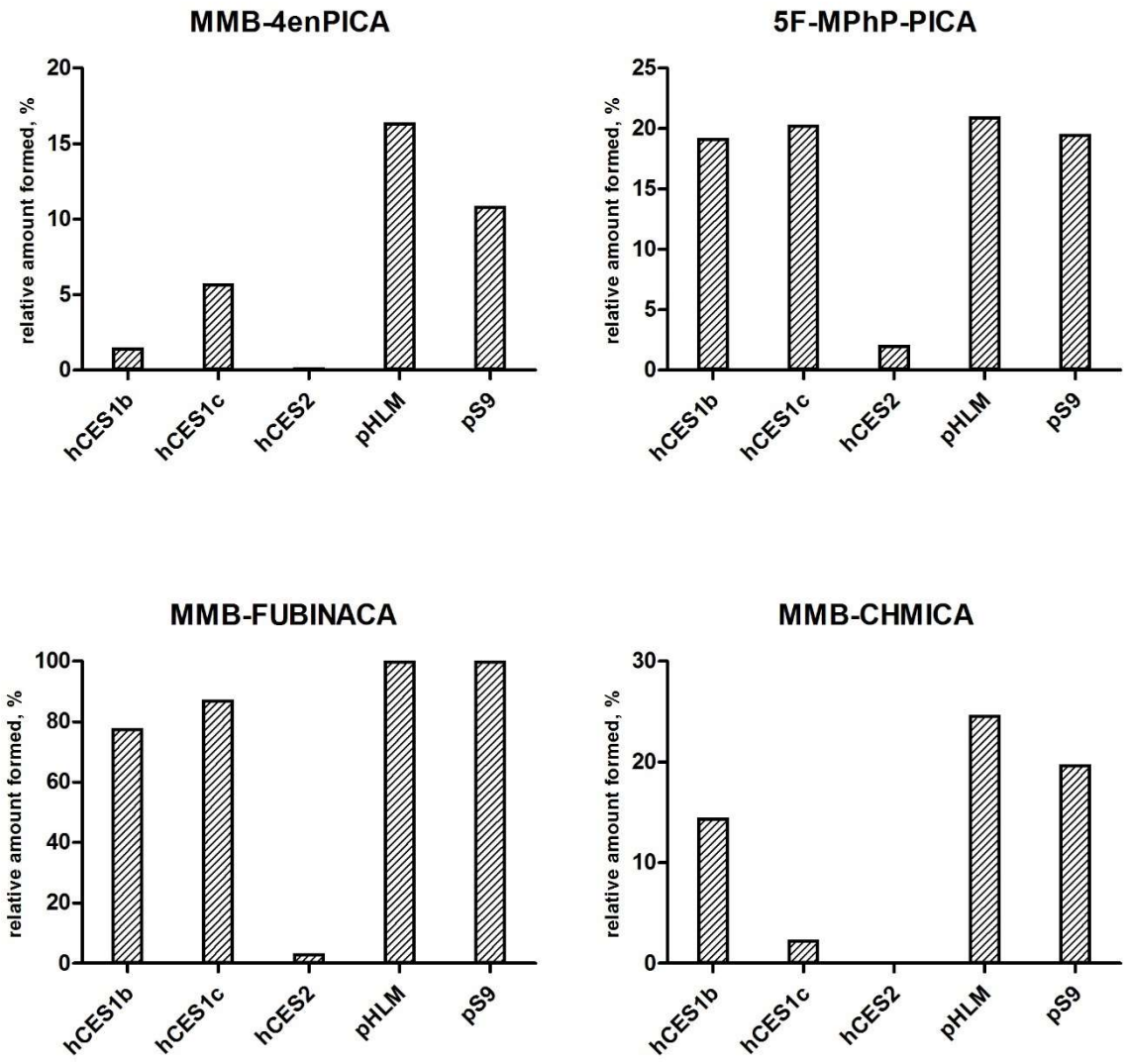

Figure 4 *Initial activity screening studies with MMB-4en-PICA, 5F-MPhP-PICA, MMB-FUBINACA, and MMB-CHMICA*

The SC with a *tert*-leucine derived structural subclass in their secondary moiety (5F-MDMB-PICA, MDMB-FUBICA, 4F-MDMB-BINACA, and MDMB-4en-PINACA) showed very low hydrolysis rates as shown in Figure 5. For hCES hydrolysis rates were under 1% and for liver cell preparations hydrolysis rate was under 5%. 5F-MDMB-PICA showed no metabolite formation at all.

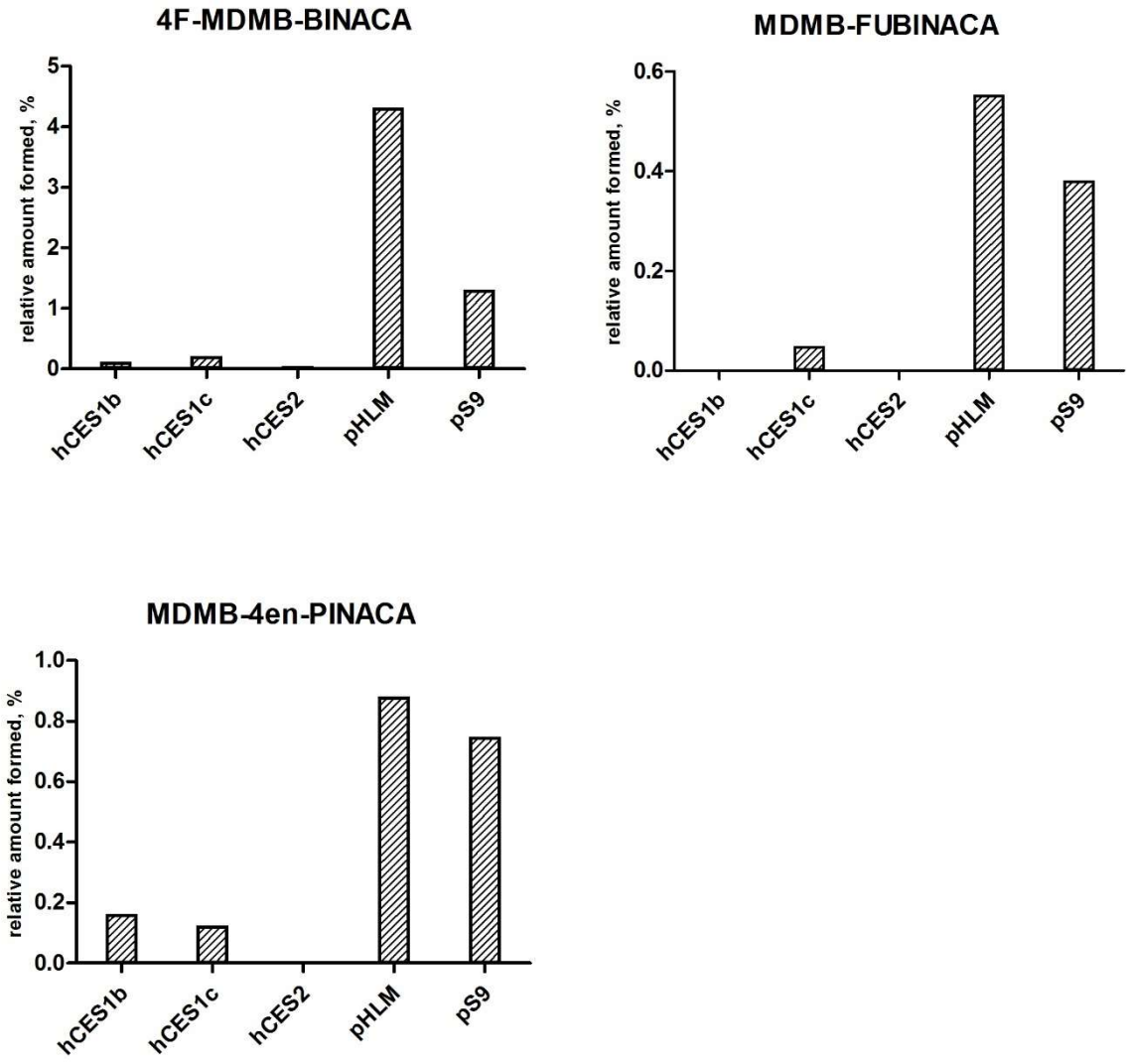

Figure 5 *Initial activity screening studies with 4F-MDMB-BINACA, MDMB-FUBINACA and MDMB-4en-PINACA*

4.1.2.2 Group 2: amide linker and terminal carboxylic acid

MBA-CHMINACA and DMBA-CHMINACA formed group 2 with an amide linker and terminal carboxylic acid. For both substrate no metabolite formation was detected.

4.1.2.3 Group 3: amide linker and a terminal amide bond

Group three included 3,5-5F-AB-FUPPYCA, 5F-AB-P7AICA, and AB-PINACA, all containing an amide linker and a terminal amide at the secondary moiety. 3,5-5F-AB-FUPPYCA and 5F-AB-P7AICA were screened according to the original experimental conditions, which were also used for all other SC, and therefore analyzed by the LC-ITMS apparatus. In these experiments no metabolite formation was detected. However, since previous studies show metabolite formation for AB-PINACA when incubated with hCES1 [16], we decided to reproduce the experiments of Thomsen *et al.* [16] which were then analyzed by the LC-HRMS/MS apparatus as herein beforementioned..

The initial screening according to the experimental procedures described by Thomsen *et al.* [16] of AB-PINACA showed a hydrolysis rate for hCES1b of 1.4% after 20 minutes. For better comparison we included pHLM and pHLS9, which both show hydrolysis rates under 5% after 20 minutes as shown in Figure 6.

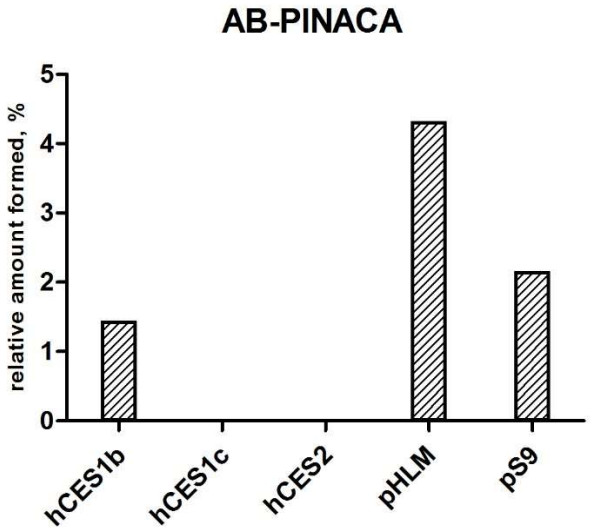

Figure 6 *Initial activity screening study of AB-PINACA according to experimental procedure of Thomsen et al. [16]*

4.1.2.4 Group 4: only amide linker

Group 4 only consisted of A-CHMINACA providing an amide linker but no terminal amide, carboxylic acid, or ester bond at the secondary moiety. In the initial activity screening no metabolite formation could be detected.

4.2 KINETIC STUDIES

After the initial activity screening studies kinetic studies were conducted with the hCES isoforms which showed the highest hydrolysis rate of at least 5%. Therefore, kinetic studies were performed as follows: test compound thebacon, kinetic studies with hCES1b, hCES1c and hCES2; MMB-4en-PICA, kinetic studies with hCES1c; MMB-FUBINACA, kinetic studies with hCES1b and hCES1c; MMB-CHMICA, kinetic studies with hCES1b; and 5F-MPhP-PICA, kinetic studies with hCES1b and hCES1c. The studied compounds were then examined for their corresponding K_m and V_{max} values.

A precondition for Michaelis-Menten kinetic modeling is that incubation time and enzyme concentration are within the linear range of metabolite formation. Examples of protein and time dependency studies with hCES1c and the test compound thebacon or the SC 5F-MPhP-PICA are shown in Figure 7.

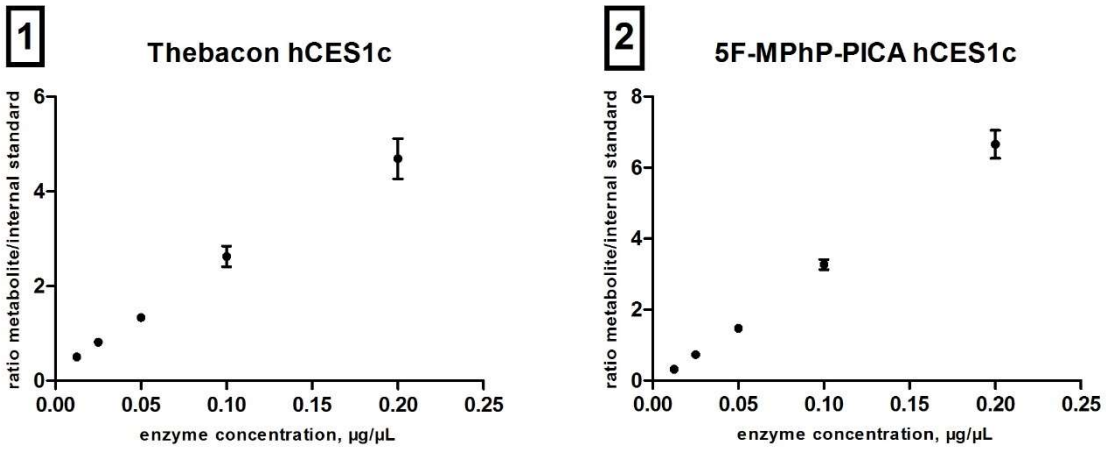

Figure 7a ***1****: protein dependency of thebacon ester hydrolysis catalyzed by hCES1c (15 minutes, 100 µM thebacon)*

2*: protein dependency of 5F-MPhP-PICA ester hydrolysis by hCES1c (15 minutes, 100 µM 5F-MPhP-PICA)*

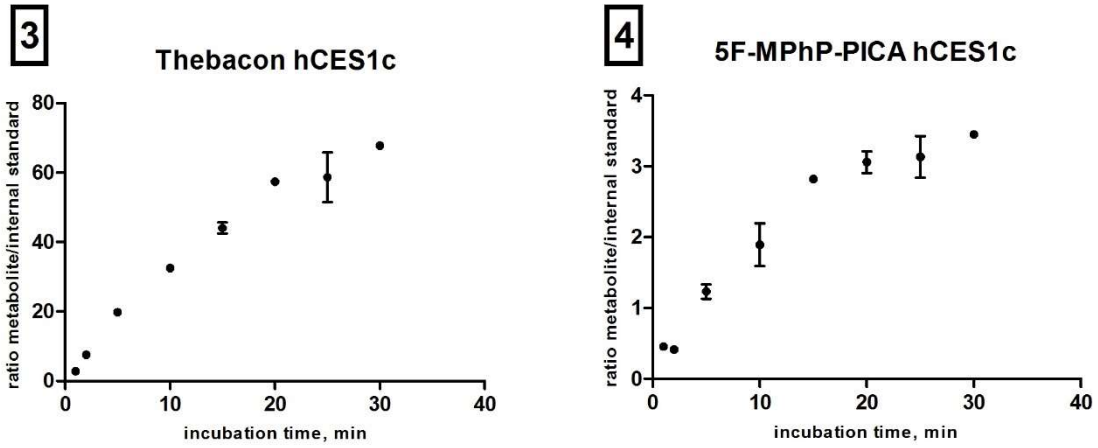

Figure 7b ***3****: time dependency of thebacon ester hydrolysis catalyzed by hCES1c (0.05 µg/µL hCES1c, 100 µM thebacon)*

4*: time dependency of 5F-MPhP-PICA ester hydrolysis by hCES1c (0.1 µg/µL, 100 µM 5F-MPhP-PICA)*

All data for determined incubation time and enzyme concentration are listed in Table 1. The resulting K_m and V_{max} values determined according to Eq. (1) for each reaction are listed in Table 3.

Table 3 *Calculated kinetic constants and kinetic parameters of the 5 studied compounds*

Substrate	Enzyme	Km, µM	Vmax, AU/min/mg
Thebacon	hCES1b	655.4 ± 207	393.6 ± 39.52
	hCES1c	1526 ± 473.6	831.0 ± 142.2
	hCES2	543.3 ± 86.75	482.2 ± 30.63
MMB-4en-PICA	hCES1c	202.5 ± 42.61	7.574 ± 0.415
MMB-FUBINACA	hCES1b	40.75 ± 15.24	70.28 ± 7.590
	hCES1c	25.65 ± 3.53	30.65 ± 0.958
MMB-CHMICA	hCES1b	39.26 ± 12.27	20.84 ± 2.244
5F-MPhP-PICA	hCES1b	88.85 ± 18.17	182.3 ± 13.01
	hCES1c	23.15 ± 4.1	73.60 ± 3.847

4.2.1 Kinetic studies of test compound thebacon

The kinetic studies of thebacon were performed with an incubation time of 5 minutes and an enzyme concentration of 0.05 µg/µL and the results are shown in Figure 8. The calculated K_m values were 655 µM for hCES1b, 1526 µM for hCES1c, and 543.3 µM for hCES2. The respective V_{max} values were 1968 AU/min/mg for hCES1b, 4155 AU/min/mg for hCES1c, and 2411 AU/min/mg for hCES2.

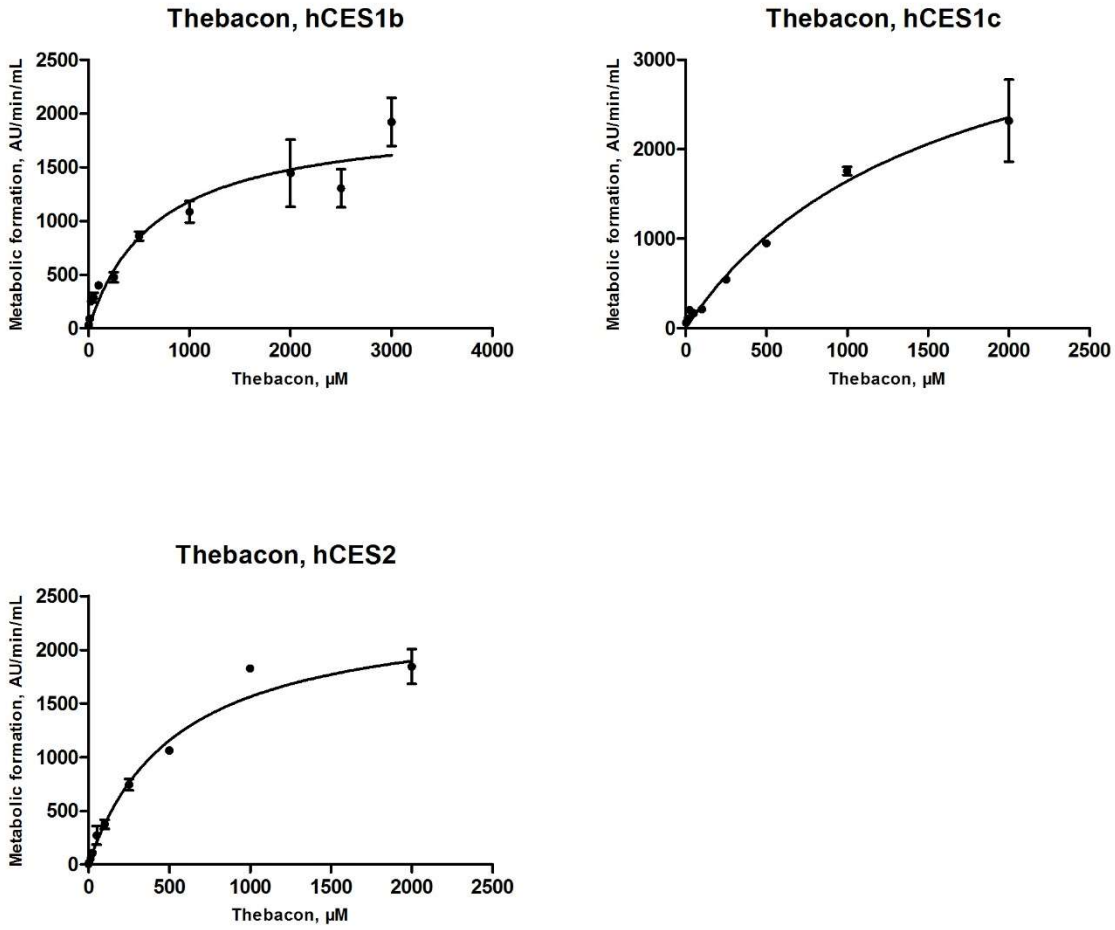

Figure 8 *Michaelis-Menten fitted plots for ester hydrolysis of the test compound thebacon catalyzed by hCES isoforms. Data points represent mean ± standard deviation (n=2). Curves were calculated by nonlinear curve fitting according to Eq. (1) (one-site binding model)*

4.2.2 Kinetic studies of synthetic cannabinoids

Further K_m values listed in Table 2 show the results of kinetic studies conducted with SC. Here K_m values ranged from 23.15 µM for 5F-MPhP-PICA and hCES1c to 202.5 µM for MMB-4en-PICA and hCES1c with incubation times reaching up to 20 minutes and enzyme concentrations of 0.1 µg/µL or 0.05 µg/µL.

More precisely the lowest K_m values were calculated for 5F-MPhP-PICA and MMB-FUBINACA and hCES1c with 23.15 µM and 25.65 µM. The respective V_{max} values were 736 AU/min/mg for 5F-MPhP-PICA and 153.2 AU/min/mg for MMB-FUBINACA. The highest K_m value was determined for MMB-4en-PICA and hCES1c as mentioned above with a V_{max} value of 75.74 AU/min/mg. Respective graphs are shown in Figure 9.

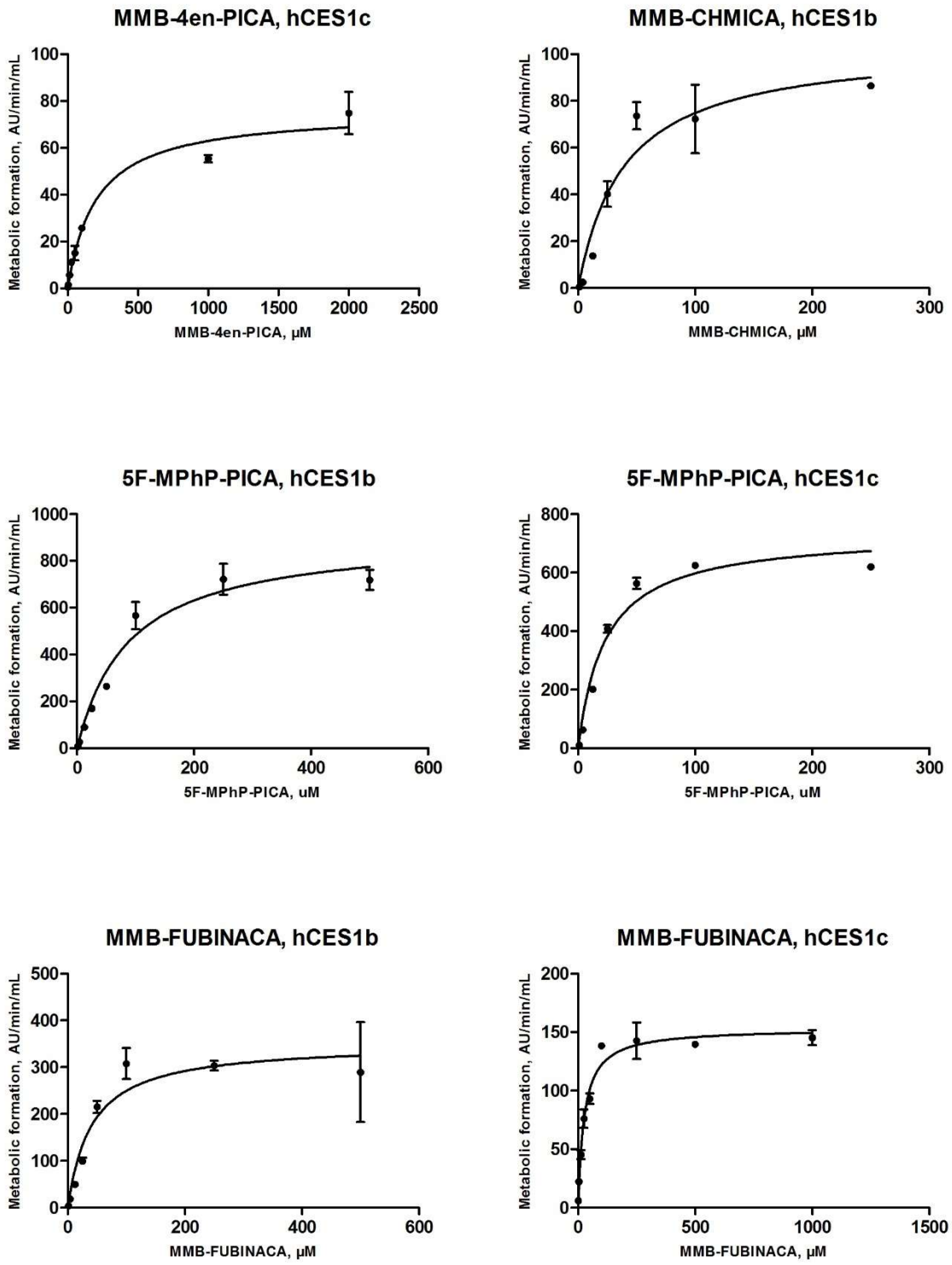

Figure 9 *Michaelis-Menten fitted plots for ester hydrolysis catalyzed by hCES isoforms. Data points represent mean ± standard deviation (n=2). Curves were calculated by nonlinear curve fitting according to Eq. (1) (one-site binding model)*

4.3 VERIFICATION OF SPONTANEOUS ESTER HYDROLYSIS

Experiments with MMB-4en-PICA were conducted, changing incubation time and the aqueous milieu in order to identify the reasons for this spontaneous hydrolysis. Results showed that there is no significant difference between samples which were incubated for 20 minutes and samples without incubation, both in phosphate buffer. In contrast to those results, dilutions in phosphate buffer and acetonitrile showed a considerable difference, with metabolite formation being more than 9-fold higher in phosphate buffer than in acetonitrile, as shown in Figure 10.

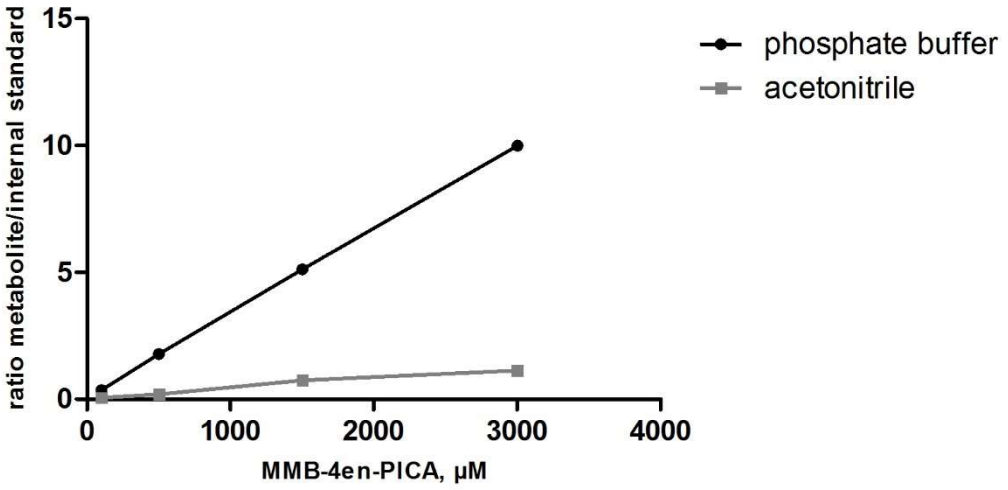

Figure 10 *Metabolite formation of MMB-4en-PICA incubations in phosphate buffer and acetonitrile*

5 DISCUSSION

5.1 GENERAL ASPECTS

The three hCES isoforms hCES1b, hCES1c, and hCES2 are phase I metabolizing enzymes known to catalyze the hydrolysis of ester and amide bonds [1] and initial activity screenings were performed with those three isoforms. However, the liver cell preparations pHLM and pHLS9 were also included, in order to better understand the metabolism of the studied compounds. Since previous studies showed that hydrolysis of SC in human plasma, which contains the four unspecific plasma esterases butyrylesterase, acetylcholinesterase, paraoxonase, and albumin [29], is highly unlikely [16, 17] we did not conduct initial activity screenings with human plasma.

Experiments by Wagmann *et al.* [30] showed that results of data analysis do not differ significantly when using peak area calculations or calibrations with refence metabolite. Furthermore, it is difficult to obtain authentic reference standards of NPS, hence data analysis was performed using peak area ratio calculations.

5.2 TEST COMPOUND THEBACON

A previous study by Meyer *et al.* [12] has already shown that thebacon is hydrolyzed by hCES1b, hCES1c, and hCES2. They observed the highest thebacon hydrolysis rate for hCES2 [12], whereas our initial activity screening indicated that the relative metabolite formation is the highest with hCES1b. Nevertheless, all three isoforms show hydrolysis rates >10% in both studies, confirming thebacon as substrate of all three isoforms and suitable test compound for further kinetic studies.

Surprisingly, Michaelis-Menten constants determined in this study were notably higher than those calculated in the previous study [12]. For instance, the K_m value for metabolite formation by hCES1b in this study was 655 µM compared to 272 µM in the experiments conducted by Meyer *et al.* [12] even though experimental conditions and analyzing methods were identical. Since a study by William *et al. [31]* stated that hCES activity decreases with a concentration of DMSO over 2%, the comparably high DMSO concentration of up to 6% in the incubations with the highest substrate concentrations might be a reason for those unexpectedly high K_m values. However, DMSO concentrations in thebacon kinetic studies conducted by Meyer *et al.* [12] was described to be under 5% and therefore comparable to the current experiments. Consequently, high DMSO concentrations cannot explain the remarkably high differences in K_m values. Hereupon, activities of hCES used by Meyer *et al.* and in our experiments were

compared and a 1.6- to 5.3-fold higher activity in enzymes used by Meyer *et al.* could be found. When corrected for the ratio of enzyme activities the K_m values are comparable as shown in Table 3. Furthermore, this difference in enzyme activity could also explain the differences between the initial activity screening of our study and the study of Meyer *et al.* Consequently, the experimental procedures and analyzing methods used in this study were successfully approved using thebacon as suitable test compound.

Table 3 *Michaelis-Menten constants corrected for enzyme activity*

	ratio A1/A2	K_m 1, µM	K_m 2, µM	K_m 2 corr, µM
hCES1b	2.4	272	655.4	274.6
hCES1c	5.3	264	1526	286.1
hCES2	1.6	166	543.3	339.6

A1 = enzyme activity in nmol/min/mg used in experiments of Meyer et al. [12]

A2 = enzyme activity in nmol/min/mg used in experiments of this study

K_m 1 = Michaelis constant calculated by Meyer et al. [12]

K_m 2 = Michaelis constant calculated in this study

K_m 2 corr = Michaelis constant calculated in this study and corrected for enzyme activity ratio

5.3 SYNTHETIC CANNABINOIDS

5.3.1 General Aspects

The difference in metabolite formation between the groups of SC was striking. Nevertheless, one similarity could be found, the SC was hydrolyzed at the terminal ester or amide bond, but no metabolite formation by amide linker hydrolysis was detected. Even though these results could be explained by the used analyzing apparatus, which might not be sensitive enough, the finding can be confirmed by several studies. If linker hydrolysis was detected, it only represented a negligible low amount of metabolite formation [17, 32-34]. For instance studies with 5F-MDMB-PICA show inconsistent findings, Mogler *et al.* [34] detected amide linker hydrolysis in pHLM and human urine samples, even if only very small amounts, while Truver *et al.* [32] did not detect an amide linker hydrolysis of 5F-MDMB-PICA, neither in pHLM nor in human urine samples.

Furthermore, all tested SC which were hydrolyzed by hCES, could be identified as substrates of hCES1b or hCES1c. Since the alcohol part of those SC, if hydrolyzed at the terminal ester or amide bond, is rather small, these results are in accordance with a previous study by Meyer *et al.* [12] stating that substrates with small alcohol groups and large acyl groups are mainly hydrolyzed by hCES1 [12].

5.3.2 Initial activity screening

Results of the initial activity screening showed that with the current experimental conditions and analyzing methodology, metabolite formation could only be detected for SC of group 1. Remarkably a striking difference was found between *tert*-leucine derived SC and other SC of group 1. While SC such as MMB-FUBINACA, being a valine-derived SC, presented a relative metabolite formation of up to 87% for hCES1c, *tert*-leucine derived SC such as 4F-MDMB-BINACA only showed a relative metabolite formation of at most 0.2% for hCES1c. For incubations with pHLM and pHLS9 similar differences could be found. For instance, in incubations with pHLM, MMB-FUBINACA showed a relative metabolite formation of 100%, whereas 4F-MDMB-FUBINACA only showed a relative metabolite formation of 4%. Those differences in metabolite formation between *tert*-leucine derived and valine-derived SC in incubations with pHLM have already been observed in a previous study by Franz *et al.* [33], who tested structurally similar SC including some of the SC tested in this study, such as MMB-CHMICA, MMB-FUBINACA and MDMB-FUBICA, suggesting that those differences in metabolite formation might be caused by different structural subclasses in the linked group.

Previous studies reported metabolite formation by ester hydrolysis in pHLM and human urine

samples for *tert*-leucine derived SC such as 5F-MDMB-PICA, MDMB-4en-PINACA, or 4F-MDMB-BINACA [17, 32-36], but only Wagmann *et al.* [17] performed a comparable initial activity screening with hCES and 4F-MDMB-BINACA, showing that 4F-MDMB-BINACA is hydrolyzed by hCES1b and hCES1c but no quantification of metabolite formation was performed. Those results as well as the results of our experiments, which all show higher metabolite formation with pHLM and pHLS9 than with hCES, indicate that hydrolysis of the terminal ester bond is an important step in the metabolism of *tert*-leucine derived SC, even if hCES doesn't seem to play an important role. Nevertheless, it has to be noted that hydrolysis in pHLM and pHLS9 is not typically used to predict the role of hCES and that an *in vitro* model cannot fully represent distribution processes which happen *in vivo*. Hence hCES might play a more important role for *in vivo* metabolism than implicated by *in vitro* studies and therefore the importance of hCES for this reaction is still disputable.

For SC of group 1 with a *tert*-leucine derived structural subclass, no previous studies of comparable initial activity screenings with hCES were found, yet further metabolism studies with 5F-MDMB-PICA, 4F-MDMB-BINACA [17, 32-34] and MDMB-4en-PINACA [35, 36] indicate that ester hydrolysis is one of the most important reactions of phase I metabolism Our experiments show that this ester hydrolysis is most likely catalyzed by hCES1 isoforms, attributing a significant role to hCES1 in the metabolism of those SC.

Furthermore, comparing the four studied SC of group 1 without a *tert*-leucine derived structural motif, hydrolysis rate for lighter substrates such as MMB-4en-PICA (342 g/mol) and MMB-CHMICA (370 g/mol) is lower than the hydrolysis rate of heavier substrates such as 5F-MPhP-PICA (411 g/mol). Additionally, heavier SC were hydrolyzed by both hCES1c and hCES1b whereas lighter SC were only hydrolyzed by one of those two isoforms.

Moreover, no metabolite formation could be detected for 5F-MMB-PICA and 5F-MDMB-PICA neither in incubations with hCES nor in incubations with pHLM. 5F-MMB-PICA and 5F-MDMB-PICA are structurally similar, the only difference being the secondary moiety which for 5F-MMB-PICA is a valine-derived structure and for 5F-MDMB-PICA is a *tert*-leucine-derived structure. Possibly these structural similarities are the reason for the undetected metabolite formation. However, both SC were proven to be hydrolized in incubations with pHLM by Franz *et al.* [33]. Another possible explanation could be the detection method (LC-ITMS-apparatus) used in this study which might be to insensitive compared to the detection method used by Franz *et al.* (LC-ESI-qToF-MS-apparatus) [33].

In conclusion especially SC without a *tert*-leucine derived structure were strongly hydrolyzed by hCES1b and hCES1c. As mentioned above hCES1 is known to be inhibited by several substances such as flavonoids or naturally occurring fatty acids [13], consequently the metabolism of hCES1 substrates can be heavily influenced. In this case SC would be metabolized at a slower rate and SC concentration in the human system would rise, causing

more toxic effects. Consequently, the results of this study should be considered when interpreting toxicological results containing these SC.

3,5-5F-AB-FUPPYCA and 5F-AB-P7AICA, both SC of group 3 containing an amide linker and a terminal amide bond at the secondary moiety, show no metabolite formation in our first initial activity screenings. Yet, amide hydrolysis of the terminal amide bond in SC has already been described in previous studies by Thomsen *et al.* [16]. They studied the metabolism of AB-PINACA and AB-FUBINACA, both SC with an amide linker and a terminal amide bond and therefore structurally comparable to SC of group 3. In order to verify our results, we reproduced the experiment for AB-PINACA with identical experimental conditions and a comparable analyzing method. Here results showed a relative metabolite formation of 1.4% after 20 minutes for hCES1b, which is roughly 4-fold lower than the metabolite formation detected by Thomsen *et al.* [16]. Since there was no significant difference in experimental conditions or analyzing method this discrepancy is most likely due to differences in enzyme activity as herein beforementioned for the test compound thebacon. Hence metabolite formation this low is probably not detectable with the Thermo Fisher Scientific LXQ which was used in this study for all other experiments.
However, other previous studies show that the terminal amide of SC of group 3 is rarely hydrolyzed in humans. Giorgetti *et al.* detected the hydrolysis product of the terminal amide of 5F-AB-P7AICA after incubation with pHLM, but in comparison to other metabolites formed the signal was rather low. Furthermore, in human urine samples the parent compound showed the highest signal compared to possible metabolites [37], demonstrating that 5F-AB-P7AICA is relatively stable and metabolized in rather low amounts in humans. These findings are in accordance to Thomsen *et al.* [16] and results of this study. Hence amide hydrolysis seems to play a minor role in phase I metabolism of group 3 SC.

SC of group 2 and group 4, hence SC which contain an amide linker and terminal carboxylic acid and the studied SC A-CHMINACA which only contains an amide linker, did not show any metabolite formation in initial activity screenings. No previous studies were found concerning the metabolism of those SC, but our results implicate that amide hydrolysis catalyzed by hCES is not a major metabolic pathway for those SC.

5.3.3 Kinetic studies

Kinetic studies were performed with MMB-4en-PICA, MMB-FUBINACA, and 5F-MPhP-PICA for hCES1c and with MMB-FUBINACA, MMB-CHMICA, and 5F-MPhP-PICA for hCES1b.

Experiments conducted with the isoform hCES1c demonstrate that the affinity to hCES1c is higher for substrates with a bigger mass than for substrates with a rather low mass. The highest affinity with a K_m value of 23.15 µM belongs to 5F-MPhP-PICA, which has the largest secondary moiety of the 4 studied SC and contains a fluor atom in its tail. Hence 5F-MPhP-PICA is 28 g/mol heavier than MMB-FUBINACA with a K_m value of 25.65 µM and shows a higher affinity to hCES1c than MMB-FUBINACA.

Kinetic studies conducted with the isoform hCES1b indicate that hCES1b shows better affinity to lighter substrates than to substrates with a bigger mass. The highest affinity with a K_m value of 39.26 µM belongs to MMB-CHMICA, which, with 370 g/mol, is the lightest of the three studied compounds. MMB-FUBINACA and 5F-MPhP-PICA with K_m values of 40.75 µM and 88.85 µM show lower affinity and are both heavier than MMB-CHMICA as mentioned before.

Those results are in accordance with previous results of Meyer *et al.* [12], if comparing their calculated K_m values.

Another aspect to be considered is the high DMSO-incubation in MMB-4en-PICA incubation mixtures, which ranged up to 4%. Reason for these high DMSO-concentration was the limited substrate solubility. As mentioned above a previous study by Williams *et al. [31]* showed that hCES activity decreases with a DMSO concentration over 2%. Therefore, it is possible that hCES activity in kinetic studies with MMB-4en-PICA was lower than in other conducted kinetic studies, in which DMSO-concentration was at 2% or lower, and that therefore the respective Km-value is falsely high.

5.3.4 Verification of spontaneous ester hydrolysis

Results of negative control incubations with MMB-4en-PICA show that there is no notable difference in metabolite formation between samples which were incubated for 20 minutes and samples without incubation. Hence incubation time does not seem to have a crucial impact on spontaneous hydrolysis.

In contrast to those results, dilutions in phosphate buffer and acetonitrile showed a considerable difference, with metabolite formation being more than 9-fold higher in phosphate buffer than in acetonitrile. A previous study concerning MMB-FUBINACA and 5F-MDMB-PICA stated that those substrates degraded to their respective butanoic acid metabolites in blood if stored at room temperature [38]. Therefore, spontaneous hydrolysis *in vivo* of MMB-4en-PICA and possibly also of other SC which are structurally similar to group 1 SC might play an important role in their respective metabolism. Other studies concerning the metabolism of group 3 SC indicate that those SC are rather stable at physiological pH [16] and that spontaneous hydrolysis *in vivo* is highly unlikely [37].

Hence, structurally different SC seem to be unequally affected by spontaneous hydrolysis at physiological pH and therefore spontaneous hydrolysis *in vivo* is more likely for certain groups of SC than for others.

6 CONCLUSIONS

The results of this study imply that hCES play an important role in the metabolism of certain groups of SC. Studied SCs were divided into four groups according to their ester and amide bonds. In general, the metabolite formed was always a product of the ester hydrolysis, the amide linker remained stable. Furthermore, after incubation with hCES metabolite formation could only be detected for SC of group 1, containing an amide linker and an ester bond at the secondary moiety. There was a striking difference in metabolite formation between group 1 SC with and group 1 SC without a *tert*-leucine derived structural subclass. For the metabolism of SC without a *tert*-leucine derived structural subclass our results imply that hCES1 plays an important role for phase I metabolism. For the metabolism of *tert*-leucine derived SC the role of hCES is rather questionable. Additionally, the weight of the substrate seems to play an important role for its affinity to the respective isoform of hCES1, hCES1b shows better affinity for larger SC whereas hCES1b shows better affinity to smaller SC. For group 3 SC, consisting of an amide linker and a terminal amide, as well as for group 2 SC containing an amide linker and a terminal carboxylic acid and group 4 SC with only an amide linker, amide hydrolysis catalyzed by hCES plays no major role in phase I metabolism. Certainly, interindividual difference du to hCES1 polymorphism cannot be excluded. Furthermore, experiments focusing on possible spontaneous hydrolysis showed that spontaneous hydrolysis might play an important role for group 1 SC and their *in vivo* metabolism.

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9 PUBLIKATIONEN

Parts of the current study will be published in an international, peer-reviewed journal soon. The tentative title is “The role of recombinant human esterases in the in vitro the metabolism of selected new psychoactive substances”

10 LEBENSLAUF

Aus datenschutzrechtlichen Gründen wird der Lebenslauf in der elektronischen Fassung der Dissertation nicht veröffentlicht.