Me pregunto: solo cuando se llega a los estudios de QM/MM recien se revisa el mecanismo de accion de las CES. En la Intro, no tiene sentido explicar eso, incluso agregando algun grafico. Al final de cuentas que el lector este al tanto de eso es util para todo el manuscrito, cuando se habla de "acilos", "alquilos", etc.

MDPI

Review

Experimental and in silico aproaches to study CES substrate selectivity

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Abstract

(margin note: Esta muy bien el abstract, solo lo siento un poco cortados en oraciones breves al inicio, con lo que hilaria un poco mas la conexion de algunas ideas.)

(margin note: hCES)

(margin note: Que tal si explicitamos?: la prediccion de metabolismo de nuevas drogas y el potencial de desarrollo de inhibidores)

Human carboxylesterases (CES) are enzymes that play a central role in the metabolism and biotransformation of diverse substances. The two most relevant isoforms, CES1 and CES2, catalyze the hydrolysis of numerous approved drugs and prodrugs. Understanding CES isoform substrate specificity is crucial for multiple research areas. Various experimental and computational methodologies have been developed to quantify CES kinetic parameters (k_{cat} and K_M) for different substrates. This review will focus in the recent advancements in these methodologies to study substrate selectivity between CES1 and CES2. Experimental measurements commonly use recombinant CES or human tissue microsomes. Chromatographic methods, such as HPLC and LC/MS, are widely employed because they allow separation of substrates from hydrolysis products. Computational approaches are typically divided into two categories: molecular docking, molecular dynamic simulation and free-energy of binding to study CES–substrate affinity, and hybrid QM/MM simulations to evaluate catalytic turnover rates. Both approaches have demonstrated high accuracy and often agree with experimental results. The advantages of integrating experimental and computational techniques are evident in several studies that clarify *(margin note: elucidates)* the structural and mechanistic basis of CES substrate selectivity. Given the biological relevance of CES-mediated catalysis, this review aims to provide a concise resource for continued exploration of CES isoform specificity and its implications for drug and prodrug design.

Keywords: Carboxylesterases; CES1; CES2; substrate; selectivity; HPLC; LC/MS; Docking; MD simulation; QM/MM

1. Introduction

Human Carboxylesterases (CES, EC 3.1.1.1) are serine hydrolase enzymes responsible for the metabolism and biotransformation of diverse endogenous and xenobiotic *(margin note: exogenous)* substances *(margin note: substrates)* containing esters, thioesters, amides, carbonates and carbamates moieties in their structures[1–3]. Based on amino acid sequence homology, human CES are classified into five isoforms (CES1–CES5). Among these, CES1 and CES2 are the most clinically relevant isoforms[4–6].

CES1 and CES2 share 47% of protein sequence identity but exhibit distinct substrate specificities and tissue distributions. Specifically, CES1 is primarily expressed in the liver, whereas CES2 is predominantly found in the intestines[1,3,4,7]. It is widely accepted in the literature that the substrate binding and hydrolysis selectivity of each isoform are *(margin note: is)* primarily determined by the size of the acyl and alkyl moieties of the respective substrate

molecular structures[4,6–8]. In this context, CES1 preferentially catalyzes the hydrolysis of substrates with smaller alkyl than acyl groups, as seen with drugs and prodrugs like clopidogrel, oseltamivir and meperidine[2,8,9]. Conversely, CES2 tends to hydrolyze compounds with smaller acyl than alkyl groups, examples of which include halperidol, procaine and flutamide[1,3,8].

Despite this general trend, several exceptions to substrate selectivity have been reported. Notably, drugs and prodrugs like irinotecan, propanil, oxybutinin and prolocaine have been shown to be metabolized by both CES isoforms with similar efficiency [8,10]. Another noteworthy example is the differential CES-mediated biotransformation rates of pyrethroid derivatives, where *cis* and *trans* isomers of permethrin exhibit distinct hydrolysis pattern by CES1 and CES2, despite having identical acyl and alkyl group sizes[11].

Given the critical role of CES-mediated catalysis in various physiological processes, understanding the structural determinants of substrate specificity among CES isoforms is crucial for several areas of research. One such area is the design of prodrugs with optimized biopharmaceutical profiles. This approach aims to avoid undesirable early biotransformation of the prodrug or to enable site-specific bioactivation targeting a particular CES isoform[2,12]. In addition, the development of specific inhibitors for CES1 and CES2 offers promising therapeutic potential for managing metabolic diseases. For instance, the CES1 inhibitor GR148672X has been explored as a candidate for treating hypertriglyceridemia, obesity and atherosclerosis[13].

In the field of medical imaging, there is growing interest in the development of fluorescent biological probes for selective detection of CES1 and CES2 activity[5,14–17]. Over recent years, the selective imaging of *in vivo* enzyme activity has emerged as a powerful method for the study of biological systems, due to its ability to provide real-time, noninvasive monitoring within living organisms[5,14–17]. In this context, the design of fluorescent probes that specifically target CES1 and CES2 offers a promising strategy for visualizing their activity in complex biological systems.

Enzymology has long been a foundational field for studying enzyme structure and function, as well as advancing our understanding of biological phenomena such as intermediary metabolism, molecular biology, and cellular signaling and regulation[18]. Early enzymology focused primarily on experimental techniques aimed at analyzing the catalytic properties and molecular specificity of enzymes[18]. The first one studies the thermodynamic and kinetic of the enzymatic reaction, measuring free-energy of reaction and activation. The second studies the specificity of different molecules for the enzymes, not only limited to the substrates, but also any other molecule that satisfy the specificity criteria for the enzyme, as is the case for potential inhibitors[18].

Due to the complexity of enzymes and the challenges associated with studying biomolecular reactions, many questions and mechanisms remain unclear. Computational enzymology, defined as the study of enzymes and the their reactions mechanisms by molecular modeling and simulation [19], has the unique potential to investigate the dynamic behavior and reactions of biomolecules at atomic resolution. This approach can address unresolved issues by complementing and interpreting findings from experimental enzymology[19–21].

Since the beginning in 1976, with the pioneering work of Warshel and Levitt (Nobel laureates in 2013), computational enzymology has rapidly evolved over the past two decades. This progress has been driven by close collaboration between experimental and computational enzymologists, enhancing our ability to explain and interpret experimental data[19–21].

Building on the symbiotic relationship between experimental and computational enzymology, this review will focus on the recent advancements in methodologies devel-

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Margin annotations (handwritten, red):

with — ing — o

larger alkyl than acyl groups

Conectaria estas dos ideas porque me parece que son parte del mismo objetivo de discusion

Me parece que hay que expandir un poquito esta seccion, con algunos ejemplos mas y detallando si es que los acilos y alquilos son considerados "grandes" o "chicos" y cual es la CES que la hidroliza. Tiene que quedar la sensacion de que la "general rule" no alcanza para pensar en drug design que contemple algun tipo de prediccion del metabolismo.

Le sumaria "la importancia de considerar interacciones entre farmacos respecto del metabolismo por CES. Hay un ejemplo de un ensayo clinico (NCT04603391) que hacen con ese ese objetivo.

Hay varios ensayos clinicos ya terminados o reclutando que son interesantes para discutir respecto de la relevancia de caracterizar el perfil de sustrato CES:
− NCT03051282
−NCT01708369
− NCT02192359: Administran celulas madre que sobrexpresan CES para activar irinotecan y tumores.
− NCT02147535: estudian el gene coding expression de CES1 y su consecuencia sobre el metilfenidato (incluir este sustrato que esta siendo muy estudiado).

to — e — In — Starting...

oped to study substrate selectivity between the two most important isoforms of human carboxylesterase: CES1 and CES2.

2. Part I: Experimental enzymology

2.1. Kinetic parameters

During the study of the enzymatic properties of different substrates, there are important kinetic parameters determine by the Michaelis-Menten fundamental equation of enzyme kinetics[18,22]. The first parameter is the **dissociation constant**, also known as Michaelis constant (K_M), which reflects the affinity between the enzyme and the corresponding substrate. A lower the K_M value indicates stronger binding within the enzyme-substrate complex [2,18,23,24]. The second key kinetic parameter is the **turnover number**, also referred as catalytic constant (k_{cat}). This value represents the number of catalytic cycles the enzyme can complete per unit of time when it is fully saturated with substrate. A higher k_{cat} value signifies greater substrate turnover and more efficient metabolism by the enzyme [2,18,23,24].

The ratio between these two kinetic parameters (k_{cat}/K_M) is known as **specificity constant**, as it reflects the ability of an enzyme to discriminate between structurally similar substrates. A higher k_{cat}/K_M ratio indicates that the enzyme shows high affinity (low K_M value) and high catalytic rate for the substrate. Because of this, the specificity constant is often used as a measure of **catalytic efficiency**, indicating if the ligand is a good or poor substrate for the enzyme[18,24].

To obtain these kinetic parameters to quantify the substrate selectivity for a specific enzyme, experimental enzymology is used to measured the progress of an enzyme-catalyzed reaction. Like any other chemical reaction, the progress can be monitored either by measuring the formation of the products or the consumption of the substrate. Reliable detection methods for product formation or substrate depletion are essential for a successful enzyme assay[18].

2.2. Enzyme sources

This section outlines the different CES enzymatic sources used in various analytical methodologies for calculating kinetic parameters. The two primary sources are *ex vivo* tissues, including purified human tissues and human microsomes, and pure recombinant enzymes (Table 1).

Cambiaria la la descripcion a algo: "Reported substrates...."

Table 1. Enzymatic sources and analytical methods used for the exploration of CES kinetic parameters on different substrates.

Juntaria en "Source"

Iria a este nivel

Substrates	Ex vivo tissue		Recombinant enzyme	Analytical method	Reference
	Human tissue	Human miocrosomes			
Irinotecan	Liver	-	-	HPLC-FL	[25,26]
Methylphenidate	Liver	-	CES1/CES2	LC/MS	[27]
Oseltamivir	-	HLM/HIM	-	HPLC-UV	[8,28]
Temocapril	-	HLM/HIM	CES1/CES2	HPLC-UV	[8,29]
Aspirin	-	HLM/HIM	CES1/CES2	HPLC-UV	[29,30]
Clopidogrel	-	HLM/HIM	CES1/CES2	HPLC-UV	[8,30]
Flutamide	-	HLM/HIM	CES1/CES2	HPLC-UV	[31,32]
Pyrethroids	-	-	CES1/CES2	FL	[23,33]
Fluorescein diacetate	-	HLM/HIM	CES1/CES2	FL	[34]
Plasugrel	-	-	CES1/CES2	LC/MS	[35]
Heroin	-	-	CES1/CES2	HPLC-UV	[36]
Cocaine	-	-	CES1/CES2	HPLC-UV	[36,37]
Oxybutynin	-	HLM	CES1/CES2	LC/MS	[38]
Prilocaine	-	HLM	CES1/CES2	HPLC-UV	[39]
Lidocaine	-	HLM	CES1/CES2	HPLC-UV	[39]
Clofibrate	-	-	CES1/CES2	HPLC-UV	[8]
Fenofibrate	-	HLM/HIM	CES1/CES2	HPLC-UV	[8,40]
Imidapril	-	-	CES1/CES2	HPLC-UV	[8]
Enalapril	-	HLM	CES1/CES2	LC/MS	[41]
Sacubitril	Liver	-	CES1/CES2	LC/MS	[42]
Anordrin	-	HLM/HIM	CES1/CES2	LC/MS	[43]

HLM: Human liver microsomes. HIM: human intestine microsomes. FL: Fluorescence

2.2.1. *Ex vivo* tissue

Since the early 2000's, the enzymatic studies of CES1 and CES2 substrate hydrolysis have relied on purified isoforms sourced from human liver. In several studies, human liver tissue is processed by homogenization and centrifugation, followed by separation of CES1 and CES2 isoforms using various chromatographic columns[25–27]. Using this method, it was determined that the produg irinotecan, along with other metabolites, is primarily bioactivated by human CES2 isoform [25,26]. *No esta indicado en la tabla*

Dejaria la "second source" para la fuente recombinante. Esto es una subfuente de la primera.

The second source of CES is human tissue microsomes, which are small vesicles derived from fragmented cell membranes, mainly endoplasmic reticulum. These human microsomes can be obtained by differential centrifugation of the corresponding tissue or purchased from different biological supply companies. Human liver microsomes (HLM) are commonly used as enzyme source for measuring metabolic stability, as they contain key metabolizing enzymes, including CES. As mentioned in the introduction, CES1 is predominantly expressed in the liver, making HLM a valuable source for studies of CES1 substrate selectivity. Following *no seria similar* similar tissue distribution patterns, human intestines microsomes (HIM) have been used as a source for CES2 in selectivity assays. Using this approach, studies have shown that the antiviral produgs oseltamivir and temocapril are preferentially activated by CES1 (HLM) over CES2 (HIM)[28,29].

2.2.2. Recombinant enzyme

Shortly after the use of human tissue as enzyme source, it became evident that a more purified version *source* of both CES isoforms were necessary to performed accurate selectivity enzymatic experiments with different substrates. Morton and Potter developed a method for cloning and expressing CES using baculovirus to infect *Spodoptera frugiperda* insect cells [44]. This technique enabled the production of recombinant CES1 and CES2, which were

then used to measure the enzymatic properties of several substrates (Table1). The hydrolysis of pyrethroids by human CES1 and CES2 have been studied using this recombinant enzyme source[23,33]. Additionally, the hydrolysis specificity of CES1 and CES2 for drugs of abuse, such as heroin and cocaine[36], as well as other drugs and prodrugs, have also been studied using recombinant enzymes (Table1)[8,38,39].

As recombinant CES enzymes became commercially available from multiple suppliers, research groups were able to continue exploring the metabolism selectivity of CES isoforms across different substrates. For example, studies on the angiotensin-converting enzyme inhibitors enalapril and ramipril showed that both drugs were selectively hydrolyzed by CES1[41]. Recombinant CES enzymes have also been used to determine the predominant role of CES isoforms in the human bioactivation of prodrugs such as sacubitril[42] and anordrin[43]. Additionally, recombinant CES enzyme have facilitated preclinical evaluations of the bioactivation rates for structurally designed prodrugs, including atorvastatin[45,46], indomethacin[47,48] and haloperidol[49].

2.3. Reported analytical methods

As mentioned earlier, obtaining accurate kinetic parameters requires a reliable analytical method to quantify the progress of the enzyme-catalyzed reaction. In this section, the different analytical methods used for the determination of the described kinetic parameters for different substrates and both CES isoforms will be described.

2.3.1. Spectrophotometry

Spectrophotometric methods have been widely used to quantitatively determine total CES activity, by measuring the absorbance of *p*-nitrophenol, produced through the hydrolysis of *p*-nitrophenyl acetate (pNPA), at 405 nm (Figure 1)[36,50,51]. This approach was applied to the functional characterization of recombinant human CES expressed in *E. coli* as an alternative method for obtaining the human enzyme[51].
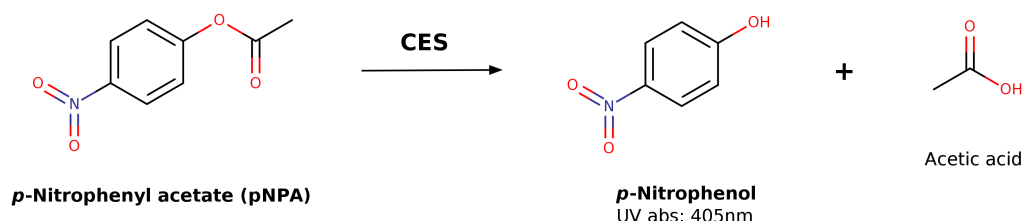
Figure 1. Hydrolysis of *p*-nitrophenyl acetate (pNPA) by CES to produced *p*-nitrophenol.

The absorbance properties of *p*-nitrophenol have also been used to evaluate the kinetic parameters of various *p*-nitrophenyl esters derivatives with CES1 and CES2[36]. The authors observed a correlation between the affinity constant (K_M) and the calculated water/octanol partition coefficients (clogP) values, concluding that the affinity of the substrates for both CES isoforms is directly related to their lipophilicity properties[36]. In addition, kinetic data for naphthyl esters derivatives were obtained by measuring the formation of naphthol at 230nm [50].

This method is simple and rapid, but it has several disadvantages. One key issue is the potential overlapping interference between substrates and products, which complicates experiments in complex biological systems. Additionally, the method requires a larger amount of enzyme, as it is performed in a UV cuvette with a total volume of 1 ml[51].

Habria que describir en que consiste el metodo.

2.3.2. Fluorescence

In contrast to the previously described analytical method, the fluorescent probe-based approach is not only simple but also highly selective and sensitive. These probes quantify CES activity by detecting changes in fluorescence intensity. Known as "off-on" fluorescent probes, they initially exhibited little or no fluorescence, but the hydrolyzed products release strong fluorescence in presence of CES.

This methodology has been used to determine the kinetic parameters of pyrethroids-like substrates containing 6-methoxy-2-naphthaldehyde. The fluorescence of this moiety is measured with an excitation wavelength of 330 nm and an emission wavelength of 465 nm[23]. In this study, it was observed that the steroisomeric centers of these derivatives presented a differential impact on hydrolysis by the two CES isoforms. Specifically, the presence of an (*R*)-enantiomer carbon adjacent to the ester carbonyl carbon resulted in a greater preference for CES2 hydrolysis than CES1[23]. These findings highlight the importance of the three-dimensional disposition of the substrate groups within the catalytic site of the enzyme for CES hydrolysis selectivity.

De nuevo remarcar que para cada ejemplo de aplicacion citado se deberia avanzar en alguna explicacion del "finding" como se hace aca.
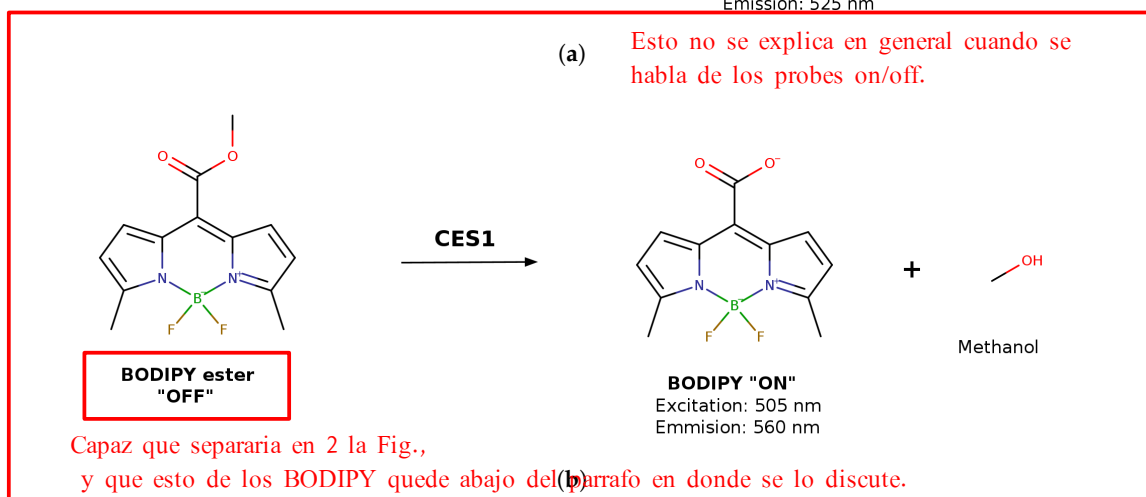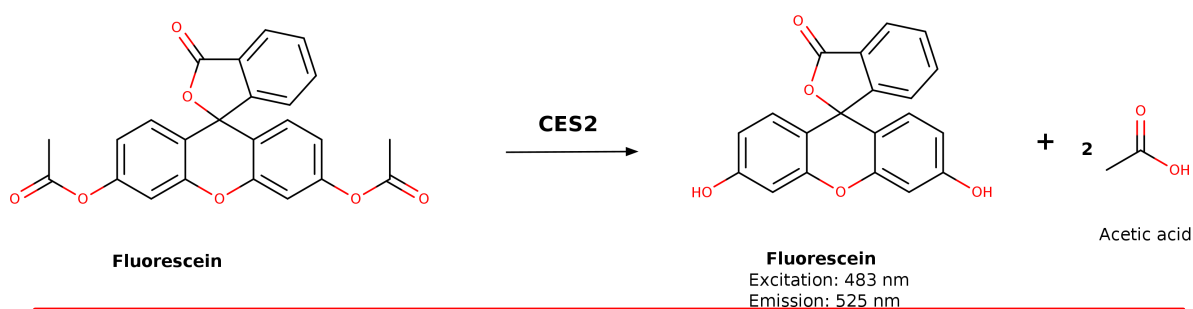
In another study, fluorescein diacetate was used as a substrate to assess CES1 and CES2 selectivity. The hydrolysis of fluorescein diacetate by the CES enzymes releases fluorescein, which was quantify *(is quantified)* at an excitation wavelength of 483 nm and emission at 525 nm. The results indicated that fluorescein diacetate is a selective CES2 substrate (Figure 2)[34].

Fluorescein

Fluorescein
Excitation: 483 nm
Emission: 525 nm

Acetic acid

(**a**)

Esto no se explica en general cuando se habla de los probes on/off.

BODIPY ester "OFF"

BODIPY "ON"
Excitation: 505 nm
Emmision: 560 nm

Methanol

Capaz que separaria en 2 la Fig., y que esto de los BODIPY quede abajo del parrafo en donde se lo discute.

(**b**)

Figure 2. hydrolysis of fluorescent probe substrates. (**a**) Fluorescein diacetate. (**b**) BODIPY ester.

The high fluorescence quantum yield and photochemical stability of BODIPY dyes make them excellent candidates for this analytical methodology. As a result, a BODIPY ester was designed as a specific substrate for CES1. The acid product formed after ~~CES~~ *CES1* hydrolysis was used to measured the kinetic parameters at an excitation wavelength of 505 nm and emission at 560 nm (Figure 2)[52].

Tener en cuenta que la cromatografia como tal es una tecnica de separacion, a la cual se le puede acoplar tanto UV como fluorescencia, con lo cual hay que buscarle el sentido aqui.

2.3.3. Chromatography

Diverse chromatographic methods have been widely used to determined the kinetic parameters of numerous substrates (e.g., drugs, prodrugs, pyrethroids) due to their high sensitivity, accuracy, rapid analysis and reproducibility. One additional advantage is the ability to separate substrate from products for precise quantification. The two most commonly used techniques are the high performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC/MS).

its — *Deberia hablar de la tecnica (its)*

Como sentido a incluir la cromatografia, me parece que estaria bueno centrar en este punto de la LC/MS que efectivamente tiene mas sensibilidad y especifidad que la a otros metodos de deteccion. Entonces capaz esta seccion la cambiaria a LC-MS/MS

In the case of HPLC, an additional detection technique is required to quantify the different ligands after chromatographic separation, with the two previously described methodologies being the most frequently used. The spectrophotometric methodology usually implement (couples) an UV-detector (HPLC-UV), as most ligands exhibit absorbance in the ultraviolet spectrum. This methodology has been widely used to study the CES kinetic parameters of various therapeutic drugs and prodrugs.

no seria "additional", por la LC no es de deteccion.

citas

To investigate substrate specificity among CES isoforms, a study was conducted on 13 compounds, including clopidogrel, clofibrate, oseltamivir, mycophenolate mofetil, procaine and temocapril, among others (Table 1), using HPLC with specific conditions for each metabolite (e.g., mobile phase, column and UV wavelength)[8]. This research group also applied the HPLC-UV method to analyzed (study) the kinetics of other drugs, such as flutamide[31,32], prilocaine and lidocaine[39]. Based on the structural characteristics of these compounds, the authors proposed the already discussed general substrate selectivity pattern for CES: CES1 preferentially hydrolyzes ligands with smaller alkyl than acyl moiety (e.g., clofibrate, lidocaine, temocapril), while CES2 favors those with larger alkyl than acyl groups (e.g., flutamide, procaine).

The metabolism of the abuse drugs cocaine and heroine by CES1 and CES2 was also studied using this methodology[36]. For cocaine, hydrolysis was monitored by quantifying the formation of benzoylecgonine and benzoic acid at 235nm. After incubation with both CES isoforms, the results showed that cocaine was exclusively metabolized by CES2, producing benzoic acid and ecgonine methyl ester. The second potential metabolic pathway, forming benzoylecgonine and methanol, was not detected[36].

Si fue detectada con CES1?

For heroine, hydrolysis was assess by monitoring the formation of 6-acetylmorphine, also at 235nm. Both CES isoforms were found to hydrolyzed heroin, with CES1 exhibiting a higher catalytic efficiency (k_{cat}/K_M) compared to CES2[36]. This result represents an exception to the general CES1 substrate specificity, as heroin has a larger size alkyl group than acyl moiety in its structure.

Porque seria un "different approach"? Estamos discutiendo la cromatografia mas que la estrategia de diseno de analogos.

A different approach was presented in several studies by Takahashi et al., in which all the investigated substrates were structurally diverse indomethacin-derived prodrugs. The formation of indomethacin, monitored at 254 nm, was used as the analytical marker to determine the kinetic parameters[47,48,53]. By synthesizing prodrugs bearing a variety of alkyl moieties, the authors were able to investigate how different structural features influence the hydrolysis behavior of both CES isoforms. Specifically, they examined: 1) the effect of the steric hindrance on the carbon adjacent to the carbonyl group, 2) the influence of electron density around the carbonyl group and 3) the chiral recognition ability between CES1 and CES2.

Lo juntaria porque la idea es enunciar el uso de la tecnica y una breve descripcion de los resultados.

Overall, the results indicated that the indomethacin prodrugs were mainly hydrolyzed by CES1, because the drug structure represents the acyl group, which in all the cases was larger than the corresponding alkyl group. However, prodrugs with aryl-containing alkyl moieties exhibited reduced or even no selectivity between CES isoforms, demonstrating that steric hindrance near the ester carbonyl carbon plays a crucial role in determining metabolic selectivity.

This research group also conducted similar studies by synthesizing prodrugs derivatives of haloperidol[49] and atorvastatin [46], using the absorbance properties of these drugs as detection methods for the HPLC technique.

Esto no es estrictamente correcto, ya la la LC-MS/MS es una combinacion de una tecnica de separacion con deteccion. Entonces no le veo sentido a decir: "does not requires an additional method..."

On the other hand, LC/MS does not required an additional method for the quantification of the desired kinetic parameters, as both substrate and product are detected directly by the mass spectrometer following their liquid chromatographic separation. This analytical technique has primarily been used to investigate the metabolic pathway of a diverse set of prodrugs.

El fuerte de la LC-MS/MS es la sensibilidad/especificidad. Entonces trataria de enfocar las revisiones en aplicaciones que hacen uso de esa caracteristica.

Interesting results were observed in the hydrolysis study of capecitabine, a carbamate prodrug of 5-fluoruracil used in the treatment of colorectal cancer[54]. The study showed that capecitabine is hydrolyzed equally by both CES1 and CES2, suggesting that substrates containing carbamate groups can be metabolized by either isoform, independent of the size of their acyl or alkyl moieties. Other prodrugs studied include plasugrel, which exhibited selectivity for CES2[35], sacubitril, selectively activated by CES1[42], and anordrin, which showed similar hydrolysis parameters with both CES isoforms[43].

In a more recent study, the hydrolysis characteristics of cocaine by CES1 were re-examined using LC/MS methodology[37]. This study revealed that cocaine is hydrolyzed by CES1, producing benzoylecgonine and methanol, which contrast with earlier findings using HPLC-UV where this hydrolysis product was not detected (Figure 3)[36]. These results suggest that LC/MS provides a more accurate analytical approach for studying cocaine hydrolysis compared to HPLC-UV.

Ojo con esto: los metabolitos son intrinsecos al proceso enzimatico, no dependen de la tecnica de estudio. Entonces me pregunto: porque perfilan productos de hidrolisis distintos? Me parece interesante el punto que estas discutiendo, pero debemos manejarlo bien para enfocarlo en la relevancia de las distintas tecnicas analiticas, que es a lo que la seccion se dedica.
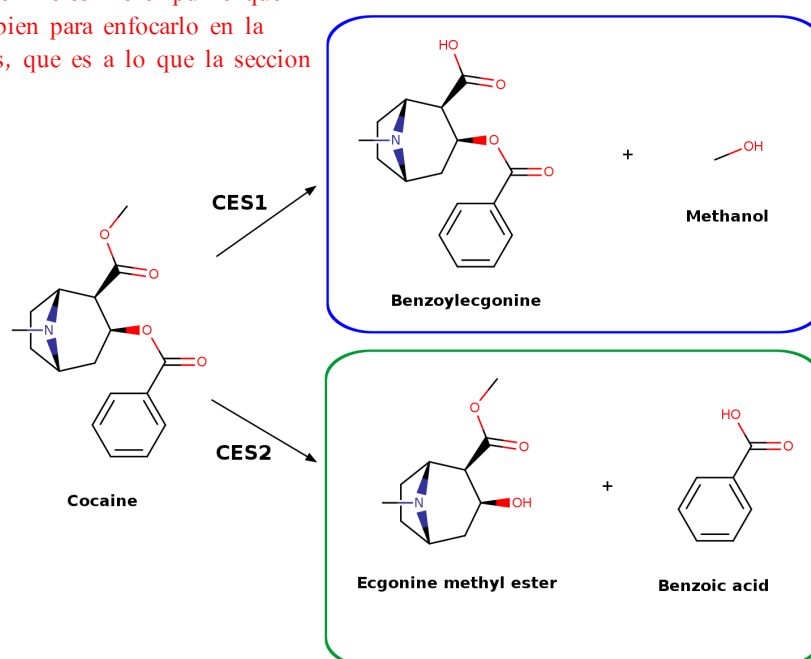

reescribit el ingles

Figure 3. Different obtained cocaine metabolic pathway from CES1 and CES2 hydrolysis experimental studies with HPLC-UV and LC/MS analytical methodologies.

This section highlights the importance of experimental enzymology in studying CES substrate selectivity. The gathered information shows that, despite the general structural rules established for substrate selectivity between CES1 and CES2, several exceptions and underlying structural properties remain unclear. Given that CES binding and hydrolysis involve subtle intermolecular interactions, computational enzymology emerges as a crucial tool for analyzing CES substrate selectivity at the atomistic level.

2.4. Experimental CES kinetic parameter databases

In the era of bioinformatics, providing reliable kinetic parameter data and effective data management systems is essential to support researchers in retrieving enzymatic information from the vast amount of biological data published annually, such as those related to CES enzymes. In this context, online databases have become invaluable tools for granting researchers access to this information. This section will focus on two of the the the most well-known and widely used online databases ~~for obtaining~~ *containing* human CES enzymatic parameters for different substrates.

2.4.1. BRENDA database

The BRaunschweig ENzyme DAtabase (BRENDA) is the oldest collection of enzyme-related data *compiled* from the scientific literature, established in 1987 at the German National Research Centre for Biotechnology in Braunschweig [55]. Today, the BRENDA website (www.brenda-enzymes.org/) is accessed by over 100,000 users each month. The site is very intuitive, allowing users to search by entering a text, such as enzyme name, ligand name, EC class, inhibitors, etc., or through structured-based queries by drawing substrate/products or ligand substructures[55].

The search for CES (EC 3.1.1.1) enzymatic information on BRENDA, yielded 2079 substrates/products and 67 natural ~~products from the database. From these ligands,~~ *substances* *Among this data,...* 849 K_M values, 550 turnover numbers (k_{cat}) and 232 k_{cat}/K_M values (catalytic efficiency) can be retrieved from diverse bibliographic sources. In addition, the reaction diagrams and references associated with these substrates ~~were~~ *are* provided.

However, two main disadvantage were noted: 1) it is not possible to filtrate information based on the organism origin of the enzyme, and 2) the kinetic information can not easily be separate by CES isoforms. Despite this limitations, BRENDA remains a valuable starting point for searchif kinetic parameters related to CES1 and CES2 substrate hydrolysis.

2.4.2. SABIO-RK database

SABIO-RK is a manually curated database containing enzymatic biochemical reactions and their kinetic parameters (sabiork.h-its.org/). ~~The authors have established that the database is a~~ *constituting a ...* valuable resource for both experimental and computational enzymology researchers[56]. Data in SABIO-RK are primarily extracted manually from the literature and stored in a structured and standardized format. The database includes essential data to describe the characteristics of biochemical reactions, the corresponding biological source, kinetic properties and experimental conditions[56].

The regular search for CES name in SABIO-RK website returned 519 entries, which is a ~~smaller~~ *less* amount of information compared to BRENDA. The SABIO-RK website offers an advance search feature that allows filtering by various conditions, such as "organisms: *Homo sapiens*", which narrows the results to 168 entries related to human CES data. Additionally, it is possible to filter the results to include only data from recombinant CES enzyme (82 entries). However, like BRENDA database, SABIO-RK also has the limitation of not separating kinetic parameters by CES isoforms. In conclusion, while SABIO-RK contains less information than BRENDA, it offers better organization and more intuitive filtering options, making it easier to retrieve relevant data.

3. Part II: Computational enzymology

3.1. Enzyme sources: structures

At the beginning of any computational molecular modeling substrate-enzyme complex study, the information regarding the structure of both, the enzyme and the substrate, is needed. The structures of the ligands are relatively easy retrieved from different biological

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Margin annotations:

A partir de aca me parece que ya lo pondria en la seccion de "Computational enzymology", ya que al fin y al cabo la bases de datos son la fuente de datasets para el modelado teorico.

"As of (fecha), the search…"

Esto es cierto si se toma en cuenta la interfaz web. Agregaria un poco de info con: Pero se puede descargar la DB, con se puede manejar la info como uno quiera. Sin embargo no se provee la version SQL, sino que se da una version en texto, lo cual implica que el usuario debe en cierta forma ser experto en el parsing de esta informac. Una alternativa son los parsers en R o en Python https://github.com/Robaina/BRENDApyrser/blob/master/docs/examples.ipynb que permiter hacer un poco de data science con la DB.

lo cual luego

No me convece el titulo, sobre todo porque queda como repetitivo con la seccion experimental. Talvez utilizaria algo del estilo: "Availability of CES1 and CES2 structural templates"

o algo del estilo.

La estuctura de los sustratos no es critica de estar disponible segun mi opinion.

En realidad no es "necesaria", sino que es en algun punto "deseada". Mas que nada porque entiendo que luego incluiras algo respecto a prediccion de CES2 y docking molecular?

databases, such as those mentioned in the previous section. On the other hand, obtaining the structures of the macromolecules (enzymes) can be more challenging. This is because enzyme structures are often distributed across different sources and databases. In this section, the ~~reported sources for obtaining the three-dimensional~~ availability of structures of CES1 and CES2 enzymes will be ~~displayed.~~ discussed.

3.1.1. CES1 crystal structures

Mas bien pondria: "CES1 structure". Y luego en el parrafo se explica que existen cristales. Lo mismo para CES2 los modelos hom.

Since 2003, different crystallographic structures of CES1 have been reported, as summarized in Table 2. The first published structures involved CES1 complexes with ligands from several categories, including metabolites of abuse drugs like cocaine and heroin (homatropine and naloxone methiodide, respectively)[57], therapeutic drugs (tacrine, tamoxifen and mevastatin)[58,59], and endogenous substrates (cholate, taurocholate and coenzyme A) [60] (Table 2). Notably, the crystal structure of CES1-tamoxifen complex revealed that tacrine interacts in the catalytic binding site of CES1 in four different binding modes[58]. These findings highlights the promiscuous nature of CES1, suggesting that its ability to hydrolyzed a variety of substrates is driven by its capacity to interact with these ligands in multiple conformations[58].

Table 2. Information related to CES1 reported crystallographic structures.

Substrates	Classification	Resolution (Å)	PDB code	Reference
Homatropine	M.L.	2.80	1MX5	[57]
Naloxone methiodide	M.L.	2.90	1MX9	[57]
Tacrine	Drug	2.40	1MX1	[58]
Tamoxifen	Drug	3.20	1YA4	[59]
Mevastatin	Drug	3.00	1YA8	[59]
Ethylacetate	M.L.	3.00	1YAH	[59]
Benzil	M.L.	3.20	1YAJ	[59]
Cholate/Palmitate	E.S.	3.00	2DQY	[60]
CoenzymeA	E.S.	2.00	2H7C	[60]
CoenzymeA/Palmitate	E.S.	2.80	2DQZ	[60]
Taurocholate	E.S.	3.20	2DR0	[60]
Soman	N.A.	2.70	2HRQ	[61]
Tabun	N.A.	2.70	2HRR	[61]
Cyclosarin	N.A.	3.10	3K9B	[62]
-	-	2.20	4AB1	[63]
-	-	1.86	5A7F	[64]
-	-	2.67	8EOR	[65]
F-3	C.I.	1.83	9KWL	[66]
F-4	C.I.	1.89	9KWM	[66]

M.L.: Metabolite ligand. E.S.: Endogenous substrate. N.A.: Nerve agent. C.I.: covalent inhibitor.

Different types of covalent ligands have also been studied through crystallographic structures. The first group to be investigated included the organophosphorus nerve agents soman, tabun and cyclosarin [61,62]. More recently, attention shifted to a second family focused on the covalent binding mechanism of serine-selective electrophilic warheads. Two crystal structures were ~~generated~~ obtained showing CES1 covalently bound to 2,2,2-trifluoroacetophenone derivatives at the catalytic serine (Table 2)[66]. Additionally, there are crystallographic structures of CES1 in the absence of substrates[63–65]. (apo form) One of these structures exhibited the highest resolution of any reported CES1 crystal structure to date (1.86 Å).

Poner entre parentesis el codigo PDB.

For conducting molecular modeling studies focused on substrate selectivity between CES isoforms, the optimal approach is to use the CES1 structure with the highest possible resolution and complexed with non-covalent ligands or in a substrate-free state. On the

other hand, it is not advisable to use the crystal structures of CES1 bound to covalent ligands. This is because the catalytic residues, along with other residues in the catalytic binding site, are ~~in conformations suitable for binding covalent inhibitors, rather than for accommodating non-covalent substrates.~~

3.1.2. CES2 homology modeling structures

The crystallographic structure of CES2 remains unresolved to this day. As a result, homology modeling is necessary to obtain the three-dimensional structure of this CES isoform in order to model the binding mode of substrates in the catalytic site of the enzyme. Different research publications have reported distinct strategies for generating the homology model structure of CES2. One of the most common methodologies involves using ~~tolls~~ tools provided by the Swiss Institute of Bioinformatics server (www.expasy.org/). In the early publications, the process of generating the CES2 model followed a two-steps procedure: first, the amino acid sequence of human CES2 was retrieved from the Swiss-Prot database, and then submitted to the Swiss-Model[67] server for fully automated protein structure homology modeling[68–70]. Over time, this methodology became more automated, allowing users to directly download pre-existing CES2 models created by other researchers via the Swiss-Model server[24,71,72].

The second approach involves generating the CES2 structure using homology modeling software. Several studies have reported the use of the open-source *Modeler* software[73] to generate the homology model of CES2 and perform subsequent molecular modeling studies on this isoform[74,75].

In recent years, *AlphaFold2*, a powerful neural network-based model methodology has been developed for predicting the three-dimensional structures of proteins[76]. Since its beginning in 2021, this machine learning approach has generated more than 200 million protein structures, all freely available for download from the AlphaFold database (alphafold.ebi.ac.uk). To the best of our knowledge, despite its accessibility and remarkable accuracy, an AlphaFold-predicted human CES2 structure has not yet been utilized for the molecular modeling of this enzyme with any substrate.

3.2. Molecular modeling methods

To analyzed the results from experimental enzymology studies of the CES-substrate complexes, a series of molecular modeling methodologies can be employed. These computational techniques ~~were selected~~ have been widely used to investigate, at an atomistic level, the kinetic parameters of different substrates for both CES isoforms. This section is organized in two subsections: 1) molecular modeling approaches (molecular docking, molecular dynamics and free-energy of interaction analysis) used to study the affinity constant (K_M) and 2) hybrid QM/MM simulations employed to ~~examined~~ study event associated to… the catalytic constant (k_{cat}).

3.2.1. Molecular modeling methods to analyzed affinity constant - K_M

~~Every molecular modeling campaign~~ Tipically, the study of the recognition and binding of CES substrates… begins with a molecular docking procedure, aimed to identify substrate conformations that yield the lowest-energy interactions with residues in the CES catalytic binding site. In ~~the~~ a subsequent stage, the CES-substrate complex is subjected to molecular dynamics (MD) simulation to characterize its dynamic behavior and stability in an explicit aqueous environment and at physiological temperature. Finally, a free-energy interaction ~~analysis~~ analyses are is performed based on the MD ~~simulation data~~ trajectories obtained for the CES-substrate complex. A small subset of studies incorporate all three of these molecular modeling methods in the investigation of CES-substrate complexes. Although a few studies incorporate all three of these molecular modeling approaches when investigating CES–substrate interactions, most rely solely on molecular docking to identify the optimal substrate conformation within the CES catalytic binding site.

The first molecular docking studies investigating the selectivity of CES1 and CES2 were conducted by the group of Vistolli et al. [68,77]. In these works, 40 known CES substrates were subjected to molecular docking protocols using two crystallographic structures of CES1 (PDB codes: 1MX9 and 1YAJ) and a homology modeling structure of CES2. Different scoring functions were calculated and correlated with the experimentally reported K_M values to develop predictive models of substrate affinity toward both CES isoforms. In both cases, the results revealed a strong correlation between K_M and the calculated lipophilic interaction scores, highlighting the central role of hydrophobic interactions, primarily attributable to the abundance of apolar residues within the catalytic binding site[68,77].

A combination of molecular docking and MD simulations have been employed to assist in the design of selective inhibitors of human CES2. These studies were focused on the design, synthesis and structure-activity relationship of glycyrrhetinic acid and benzofuranone derivatives (Figure 4)[69,78]. In the first work, molecular docking was performed using the most active and selective glycyrrhetinic acid derivative against both CES isoforms to elucidate its 1000-fold preference for CES2 over CES1[69]. The results indicated that this derivative displayed a greater number of hydrogen bond interactions with residues in the CES2 catalytic binding site residues than in CES1. In addition, the conformation adopted by the inhibitor in CES1 complex was positioned farther from the catalytic serine residue than in the CES2 complex[69]. In the second study, a similar molecular docking analysis was performed for the most active and selective benzofuranone derivative against both isoforms. The results were consistent: the inhibitor exhibited more favorable interactions and a higher number binding site contacts with CES2 residues compared to CES1[78]. Furthermore, MD simulation and free-energy decomposition analyses of the CES2-inhibitor complex revealed that the complex remain stable over 50 ns of simulation and that the hydrophobic interactions played a major role in the binding of the inhibitor to CES2[78]. As shown in Figure 4, both selective CES2 inhibitors share the presence of a carboxylic acid group in their molecular structures.

Margin annotations (handwritten, red):

Early…

were designed to explore

Estos trabajos son del 2010, y una busqueda rapida muestra estudios anteriores de docking sobre CES. Mas bien enfocaria en los reviews o trabajos mas representativos y recientes de docking sobre CES.

Nunca de hablo de este residuo, lo que evidencia que es necesario en etapas tempranas revisar el mecanismos catalitico de las CES.

Dado que se destava este punto, me pregunto que implica la presencia de grupos acidos carboxilicos para la selectividad?
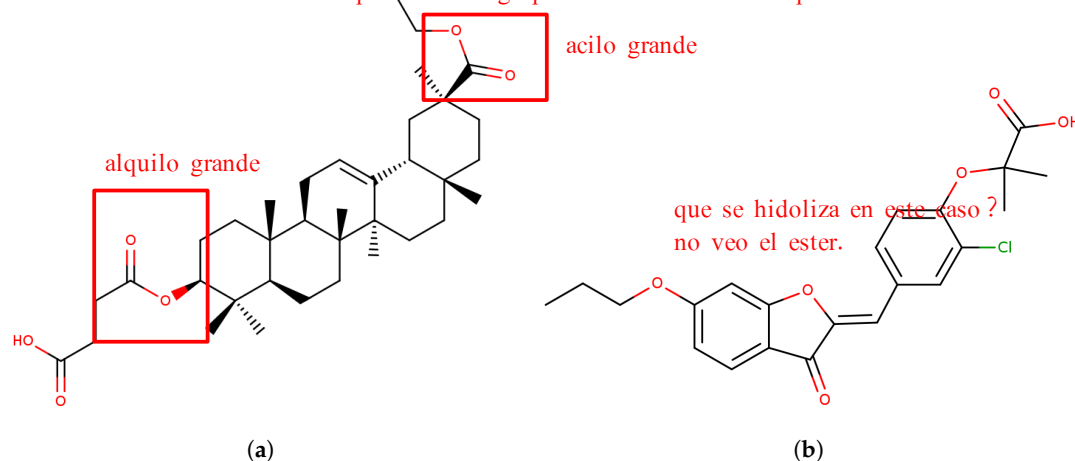

Figure 4. Molecular structures of CES2 selective inhibitors. (**a**) Glycyrrhetinic acid derivative. (**b**) Benzofuranone derivative.

Figure annotations (handwritten, red):

acilo grande

alquilo grande

que se hidoliza en este caso? no veo el ester.

In a more recent study[24], molecular docking, MD simulation and free-energy interaction decomposition analyses were performed on two families of previously studied substrate families: five *p*-nitrophenyl ester derivatives[36] and two pyrethroid stereoisomers[23], using both CES1 and CES2. Consistent with earlier findings, hydrophobic interactions were found to contribute substantially to the CES-ligand free-energy of interaction, correlating well with experimentally determined affinity constants (K_M)[24]. An additional key ob-

En realidad me imagino que para anticipar si una molecula es buen o mal sustrato de CES, se necesita verificar si la afinidad es alta y origine una baja KM, pero ademas que el modo de union que se asocia a esa alta afinidad sea cataliticamente productiva, es decir que implique el posicionamiento del grupo a hidrolizar en las cercanias de la serina catalitica y ocupando el oxianion hole.

~~servation was that the increase~~ **higher** binding cavity volume of CES2, relative to CES1, enabled one pyrethroid stereoisomer to adopt a distinct interaction pattern and thereby maintain a higher affinity (lower K_M) for CES2 ~~compared with its corresponding~~ **compared to the other** stereoisomer. Overall, the substrates analyzed in this work formed the most favorable interaction patterns with residues in the respective CES catalytic binding sites, resulting in the highest attainable affinity for each isoform[24].

3.2.2. Hybrid QM/MM simulation to analyzed catalytic constant - k_{cat}

Lo sacaria del titulo y explicaria en el texto

The study of enzyme-ligand catalytic constant through computational chemistry *no se si es una "constante catalitica enz:lig"* requires specialized methodologies to analyzed the free-energy of activation associated with the enzyme-catalyzed hydrolytic reaction. The most commonly employed approach for this purpose is hybrid QM/MM simulation. In this method, the ligand and the catalytic residues directly involved in the hydrolysis reaction are modeled using quantum mechanics (QM), while the remaining enzyme residues are described with a molecular mechanics (MM) force field.

Esto es lo que pienso es mas util en la introduccion para que quede claro el mecanismo catalitico.

The general hydrolysis mechanism of esterases, such as CES, is well established and involves two consecutive reaction steps. First, the acylation step, where the carbonyl carbon of the substrate is attacked by the hydroxyl moiety from the catalytic serine, leading to the formation of an acylated serine intermediate and the release of the alkyl group of the substrate. Second, the deacylation reaction, where the carbonyl carbon of the acylated serine is attacked by the oxygen of a water molecule, yielding the caboxylic acid portion of the substrate and regenerating the free serine residue, thereby allowing a new catalytic cycle to begin (Figure 5)[3,24,37].

1) Acylation

2) Deacylation

En el texto, donde se mencionan estas etapas, habria que citar la Figura.

Figure 5. Acylation and deacylation reaction pathways for CES1 and CES2 catalyzed hydrolysis of the ester ligands. *p*- nitrophenyl ester derivatives were used as example. Reactant state (RS), first transition state (TS1), intermediate (INT), second transition state (TS2) and product state (PS).

There **are** relatively few reported ~~reported~~ studies employing hybrid QM/MM simulation**s** for substrates hydrolyzed by human CES, likely because of the greater complexity of this methodology compared with the molecular modeling methodologies described in the previous section. Given the importance of human CES in the metabolism of abuse drugs, several

works have investigated the hydrolysis mechanism of cocaine by CES1 and CES2. The first study reported the hydrolysis mechanism of cocaine by CES1 using hybrid QM/MM simulation alongside the experimental determination of the corresponding kinetic parameters (K_M and k_{cat})[37]. The QM region was parameterized with the semi-empirical SCC-DFTB method, while the MM region with CHARMM27 force field. The reaction was modeled using an umbrella sampling approach, where the reaction coordinate (RC) was defined as a linear combination of two covalent bond distances: one forming and one breaking. The simulations indicated that CES1 catalyzes the hydrolysis of cocaine to benzoylecgonine and methanol (Figure 1) through a single-step acylation reaction followed by a single-step deacylation stage, each showing a distinct transition state (TS). The combined free-energy profile of both reaction steps revealed that the acylation TS is the rate-limiting reaction, with a free energy barrier of 20.1 kcal/mol. This result is in close agreement with the experimental free-energy barrier, derived from the catalytic constant, which was 21.5 kcal/mol, demonstrating the high accuracy of the hybrid QM/MM methodology employed in this study.[37].

A second article reported a study similar to the previous one, exploring the hydrolysis of cocaine by CES2 to produce ecgonine methyl ester and benzoic acid (Figure 1). In this study, the QM region was parameterized using the semi-empirical PM6 method, and the MM area was modeled with ~~an Amber18~~ *the ff99SB* force field (ff99SB). An umbrella sampling approach was again employed to follow the two-steps hydrolysis reaction, in which two covalent bond distances, one bond forming and one bond breaking, were used as reaction coordinates. The resulting 2D-PMF profiles for the full catalytic cycle showed that each reaction stage proceeds through a tetrahedral intermediate structure and involves two transition states. Among these, TS_4 (associated with the formation of benzoic acid) was identified as the rate-limiting step of cocaine hydrolysis by the CES2, displaying an activation free-energy barrier of 19.5 kcal/mol. This lower energetic barrier respect to CES1 is consistent with previous experimental findings that reported a higher turnover number for CES2 than for CES1.

In the previous section, a study involving two families of substrates, *p*-nitrophenyl esters and pyrethroid stereoisomers, in complex with CES1 and CES2 was described. In this work, two *p*-nitrophenyl ester derivatives and two pyrethroid stereoisomers were subjected to hybrid QM/MM simulation to analyzed their hydrolysis by both CES isoforms[24]. The QM/MM simulation protocol followed was similar to the earlier study of cocaine with CES1[37]; the QM region was parameterized using the semi-empirical SCC-DFTB3 method, while the MM region was modeled with Amber20 force field (ff14SB). As in the previous example, the study employed an umbrella sampling approach; however, in this case, four covalent bond distances, two bonds forming and two bonds breaking, were combined linearly using a linear combination of distances (LCOD) to define the reaction coordinate (RC).

Across all modeled reactions, substrate hydrolysis by both CES isoforms proceeded through a single-step [*concerted?*] acylation stage followed by ~~a single-step~~ [*another concerted*] deacylation stage, each involving one transition state (TS). Analysis of the results showed that the rate-limiting step for each substrate corresponded to the TS associated with the highest molecular steric hindrance encountered during the course of the hydrolysis, acylation or deacylation stage[24]. The authors concluded that the CES selectivity is not solely determined by the molecular size of the alkyl or acyl groups of the substrates, but instead arises from a more complex scenario governed by the initial conformation of the ligand within the CES binding site[24].

Overall, all studies discussed in this section showed a good correlation between the experimental catalytic constant (k_{cat}) and the calculated free-energy of activation from the

rate-limiting TS reaction stage, demonstrating the reliability of the hybrid QM/MM methodology. This was observed despite the differences in the proposed hydrolysis mechanisms, which involved either two or four transition-state structures.
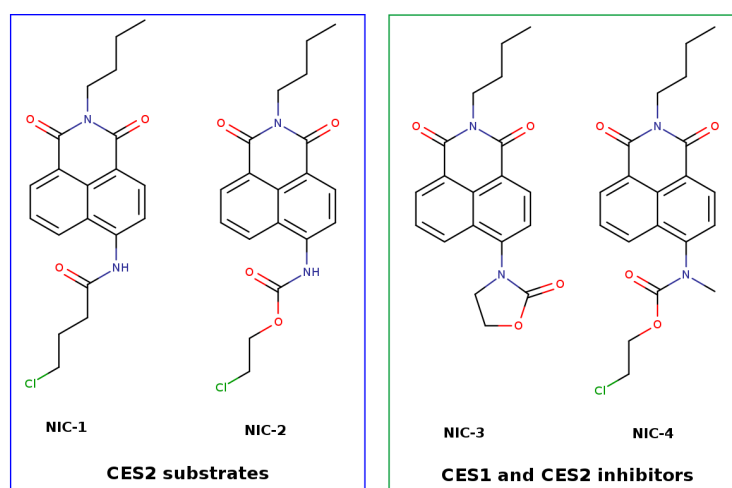
4. Part III: Combination of experimental and computational enzymology

This final section of the article is devoted to studies that combined experimental and computational methodologies to investigate the hydrolysis properties of various substrates by both CES isoforms.

In the first study, the hydrolysis of fenofibrate by CES1 and CES2 was examined to identify the primary enzyme responsible for its metabolization in humans[40]. The kinetic parameters were determined by measuring the formation rate of fenofibric acid (the hydrolytic metabolite of fenofibrate) through HPLC-UV in the presence of human microsoms and recombinant CES1 and CES2. These results exhibited that the affinity constant was slightly higher for CES1, but the major difference was observed in the catalytic constant, with CES1 exhibiting a substantially faster reaction rate than CES2[40].

The authors performed a molecular docking study of fenofibrate within the binding site of both CES isoforms. The docking results showed that fenofibrate adopted a more favorable binding pose and received a better docking score into the catalytic binding site of CES1 than CES2, consistent with the experimental affinity results. Hybrid QM/MM simulations were not performed in this publication[40]. This study provides useful insight into the factors underlying the pronounced differences in fenofibrate hydrolysis between the two CES isoforms.

The second study, reported the design and CES selectivity of a family of four fluorescent substrates derived from a naphthalimide scaffold [17]. Enzymatic assay was monitored by measuring the emission intensity of the hydrolysis product at 520 nm (excitation 450 nm) after incubation with CES1 and CES2. Only the two derivatives containing amide or carbamate moieties (NIC-1 and NIC-2, figure 6) underwent specific hydrolysis in the presence of CES2. In contrast, the other two carbamates in which the nitrogen was fully substituted with carbon atoms (NIC-3 and NIC-4, figure 6), exhibited inhibitory effects of both CES isoforms.

Figure 6. Structure of the fluorescent substrates derivatives of naphthalimide (NIC)[17].

To investigate the shift in activity from CES substrate to inhibitor, the binding properties of NIC-4 toward CES1 were analyzed using molecular modeling methods with CES1 and compared with the known substrate *p*-nitrophenyl acetate (pNPA)[17]. Molecular docking followed by MD simulations, revealed that both NIC-4 and pNPA displayed simi-

lar interaction patterns with the residues of the CES1 binding site. In addition, a steered MD simulation was performed in which the structures and the binding site residues were parameterized with SCC-DFTB method, while the remaining part of the enzyme with Amber16 ff14SB force field. The reaction coordinate was defined as the distance between the catalytic serine oxygen and the carbonyl carbon of the substrate, representing the nucleophilic attack in the hydrolysis reaction. During the simulation of pNPA hydrolysis by CES1, the proton transfer from the catalytic serine to the nitrogen atom of the catalytic histidine occurred spontaneously, following the normal acylation mechanism with an estimated energetic barrier of 20 kcal/mol. In contrast, during the simulation of NIC-4 with CES1, this proton transfer did not occur, because the methyl group positioned near the potential nucleophilic center of NIC-4 (Figure 6) sterically blocked the transfer pathway, forcing the serine hydroxyl group to orient away from the histidine imidazole. These molecular modeling results enable the authors to explained the mechanism of CES inhibition induced by NIC-4[17].

> Aca esto explica lo que me pregunte antes. Es un buen caso para destacar la importancia de comprender a nivel atomistico los enventos asociados al funcionamiento de las CES, tanto para la prediccion de substratos como para el diseño de inhibidores.

5. Conclusions

The objective of this article was to highlight the importance of combining experimental and computational techniques to elucidate substrate selectivity between the two major human CES isoforms: CES1 and CES2. The first section showed that chromatographic methodologies (HPLC and LC/MS) are widely used by different research groups for the experimental determination of CES kinetic parameters (K_M and k_{cat}) for a variety of substrates. In some cases, this methodology is complemented by spectrophotometric techniques, such as HPLC-UV, to measure enzymatic activity. The corresponding kinetic data and experimental methodologies can be found in several freely accessible enzymatic databases, including BRENDA and SABIO-RK.

The second section summarized the computational strategies employed to model substrate affinity, through molecular docking, MD simulation and free-energy of binding analysis, and catalytic turnover numbers, through hybrid QM/MM simulation, for the studied CES-substrate complexes. These computational methodologies demonstrated high accuracy and provided results consistent with experimentally reported data.

The advantages of integrating experimental and computational approaches became clear in the examples discussed in the final section, where the combined use of both methodologies enabled the elucidation of structural factors underlying substrate selectivity toward specific CES isoforms.

This collaborative strategy should be more widely adopted to accelerate advances in understanding CES substrate selectivity for future compounds. Given the biological relevance of CES-mediated catalysis, further investigation into the structural determinants governing isoform specific substrate recognition remains a significant scientific challenge, offering valuable insights for the rational design of drugs and prodrugs with optimized biopharmaceutical properties. It is hoped that this article will serve as a useful resource to support continued exploration in this field.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.; methodology, X.X.; software, X.X.; validation, X.X., Y.Y. and Z.Z.; formal analysis, X.X.; investigation, X.X.; resources, X.X.; data curation, X.X.; writing—original draft preparation, X.X.; writing—review and editing, X.X.; visualization, X.X.; supervision, X.X.; project administration, X.X.; funding acquisition, Y.Y. All authors have read and agreed to the published version of the manuscript.", please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

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Written informed consent for publication must be obtained from participating patients who can be identified (including by the patients themselves). Please state "Written informed consent has been obtained from the patient(s) to publish this paper" if applicable.

Data Availability Statement: We encourage all authors of articles published in MDPI journals to share their research data. In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Where no new data were created, or where data is unavailable due to privacy or ethical restrictions, a statement is still required. Suggested Data Availability Statements are available in section "MDPI Research Data Policies" at https://www.mdpi.com/ethics.

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Abbreviations

The following abbreviations are used in this manuscript:

MDPI	Multidisciplinary Digital Publishing Institute
DOAJ	Directory of open access journals
TLA	Three letter acronym
LD	Linear dichroism

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