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In Vitro Characterization of Fluorogenic Chemical Tools to Study Human Carboxylesterases (CESs)

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BY

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Abstract

Human carboxylesterases (CESs) are enzymes that are responsible for the metabolism of many important pharmaceuticals. Although CESs are key players in the hydrolysis of many ester-containing drugs, they remain understudied. Our group hypothesizes that this is primarily because there are few methods capable of reporting activity in live cells. Here, I report a new series of fluorogenic chemical tools to study the CES activity of one of the two major CESs in humans, CES1, in live cells. MCP-Me, MCP-Et, and MCP-iPr utilize the same carbonate group of a previously developed chemical tool in our group, FCP-1, and work to mimic the substrates of common drugs to study CES1 activity *in vitro*. By improving methods used to study CES1 activity, we can improve the individual efficacy of pharmaceuticals by ensuring prescribed drugs are compatible with the patient's metabolic enzymes activity.

Introduction

Carboxylesterases (CES) are a family of serine hydrolases that carry out the metabolism of many xenobiotic and endogenous esters within the body.[1-10] Their role is essential to proper bodily function. Five human CES isoforms are known; however, CES1 and CES2 are of particular interest.[2,5,12-15] These two serine hydrolases are responsible for the hydrolysis of most ester-containing pharmaceuticals in humans (Figure 1).[1,2,7,9] While these enzymes are similar, they possess distinct differences in their substrate preference.[6,14-17] CES1 prefers molecules with a large acyl group and a small alcohol, while CES2 favors substrates that contain a large alcohol and a small acyl group.[1,2,16,18-20]

Both CES1 and CES2 are crucial to the metabolism of many drugs; however, my primary focus is CES1.[2] This is due to data showing that differences in CES1 activity have been recorded to directly influence the success of patients treated with CES1-metabolizing drugs.[1,2,7,8,11] In

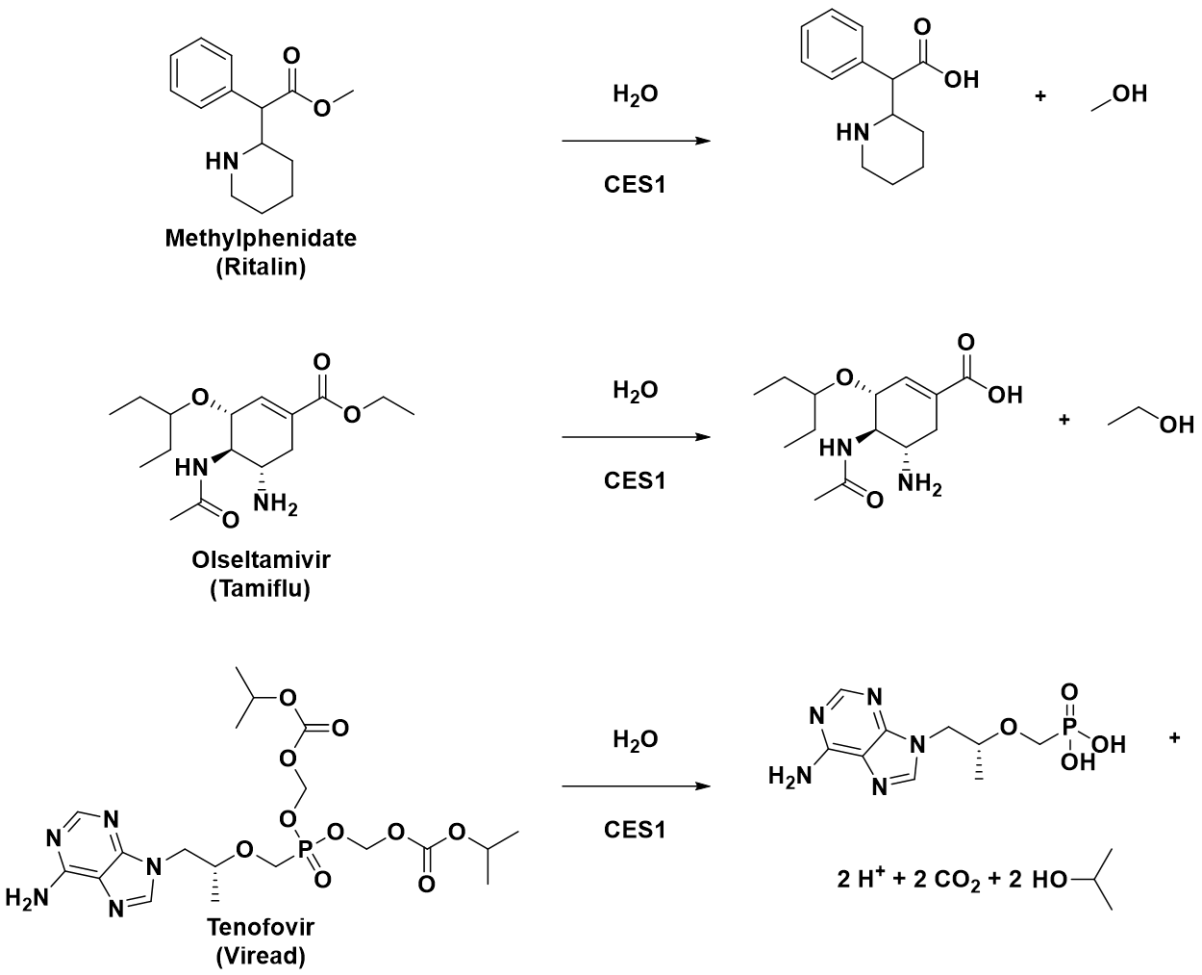

Figure 1. CES1 metabolism of common drugs containing **A.** methyl ester **B.** ethyl ester **C.** isopropyl ester.

comparison to other enzymes involved in drug metabolism, CES1 proves to be relatively understudied.[1,2] This is problematic due to the disparity of CES1 activity among humans. Activity differs due to various factors, such as single nucleotide polymorphisms (SNPs), alternate RNA splicing, and drug-drug interactions, so CES1 activity cannot be assumed to be the same between individuals.[1,7,9,11,15,16] However, CES1 activity is not regularly assessed in the treatment of patients. This is especially troublesome due to the frequent use of CES1-substrate esters across multiple drug classes including common and important drugs such as methylphenidate (Ritalin), olseltamivir (Tamiflu), and tenofovir (Viread) (Figure 1).[1,4,7] This demonstrates the urgency and importance of why CES1 activity must be further studied. Yet only few methods exist to study CES1 activity.[1,53]

The traditional method of studying CES1 activity in live cells involves the use of a known drug that metabolizes CES1 followed by analysis via chromatographic methods often paired with mass spectrometry.[1,26,27] While this method is functional, it is not ideal. Chromatography and mass spectroscopy are time consuming and provide limited information.[1] This has led us to ask a central question: how can we simplify this process while gaining more information? We hypothesized that fluorescence microscopy could help us to achieve this.[1,2,28-35] Fluorescence microscopy offers many advantages over traditional methods, one of which is that it allows the user to view enzyme activity in real time.[1,49] This allows for evaluation of the full chemical interaction of the protein of interest with other factors, as well as insight into any barriers that may be inhibiting the reaction. Fluorescence microscopy also can be utilized in solution, which is conducive to preliminary *in vitro* analysis and characterization. In addition, fluorescence microscopy is also highly sensitive, which minimizes the error produced by undetected activity in contrast to traditional methods.

Fluorogenic probes have been previously developed to study CES1 activity; however, many are poorly characterized limiting their utility to study CES1 in live cells.[1,37-39] The best characterized probes require an intricate synthesis process and fine tuning of their electronic properties to be able to report on CES1 activity.[1,2] In response to these issues, our group designed FCP-1, a more accessible fluorogenic chemical tool, that is able to be hydrolyzed by CES1 in live cells (Figure 2).[2] This has simplified the process by allowing activity analysis by fluorescence microscopy and has decreased the time it takes to measure CES1 activity changes. FCP-1 works by presenting as a CES1 preferred substrate that is metabolized via CES1 ester hydrolysis into the fluorescent compound fluorescein.

While this probe is a significant advance in the area, FCP-1 is only a drug-substrate mimetic for ethyl ester drugs.[2] Therefore, our lab has sought to create fluorescent probes that

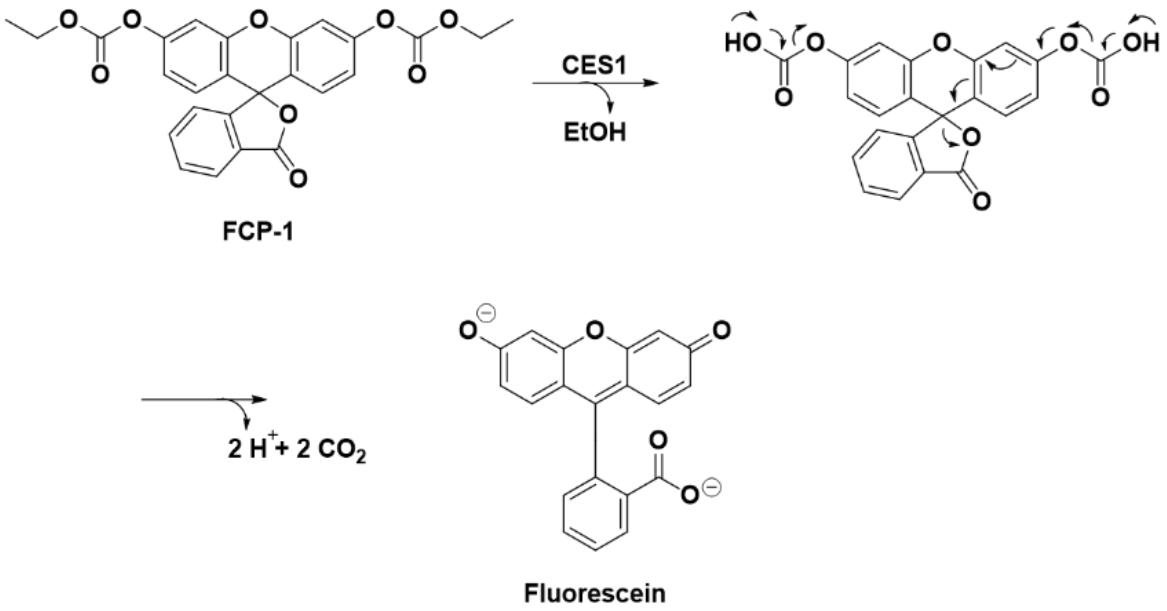

Figure 2. FCP-1 metabolizing via CES1 to produce fluorescein.

mimic the esters and carbonates of more drugs. Recently, we have developed a series of new probes based on 3-O-methylfluorescein (MOF) that aim to address this problem (Figure 3). These probes have been designed with specific drug structures in mind, with each probe tailored to mimic a specific known CES1-substrate drug. We believe the MCP series will have the capacity to allow more understanding regarding the factors that influence CES1 activity for the drugs they mimic due to their different structures. The MCP series will also allow us to analyze drug-specific interactions that are currently understudied. However, before we can deploy our MCP series in live cells, we must fully characterize their properties *in vitro*. The first characterization that must

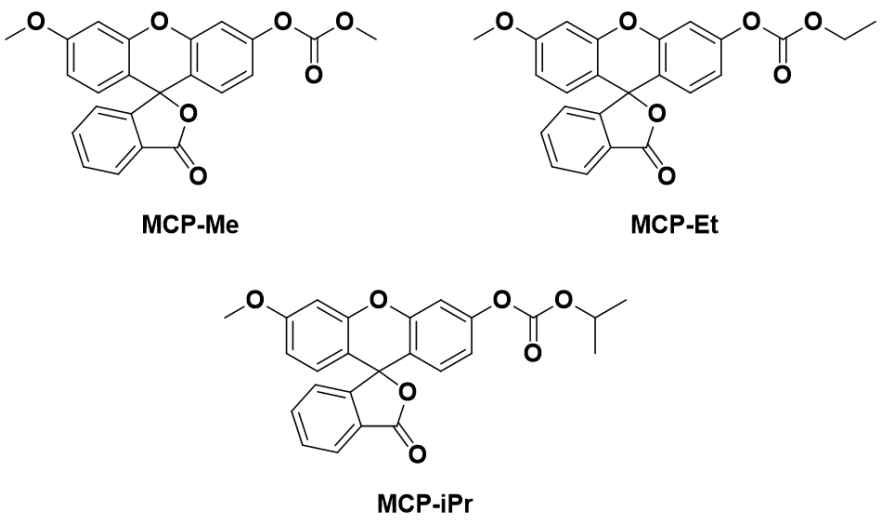

Figure 3. Structures of MCP series fluorescent probes for CES1.

take place is to ensure that the MCP probes interact as expected and specifically with CES1.[1,2,5,41-43] I will achieve this by determining their fluorescence properties and stability in solution. Once this is established, we can determine their interaction with CESs *in vitro* and in live cells.

Experimental

General materials and methods: Unless otherwise noted, all purchased reagents were used as received without further purification. Millipore filtered water was used as the water source for all experiments unless otherwise noted. All fluorescence experiments were carried out using an Agilent BioTek Synergy H1 Hybrid Multi-Mode Reader equipped with Variable Bandwidth Monochromators and Red Extended PMT (Santa Clara, CA). MOF, MCP-Me, MCP-Et, and MCP-iPr were previously synthesized by other students. All error bars are +/- standard deviation.

Fluorescence spectroscopy of MCP-Me, MCP-Et, and MCP-iPr: Fluorescence spectra were recorded using 1 μM MOF, MCP-Me, MCP-Et, or MCP-iPr in 1X PBS in a 96 well Greiner Bio-One black μclear bottom microplate (Monroe, NC) and MOF, MCP-Me, MCP-Et, and MCP-iPr were excited from 400 to 525 nm with the emission recorded at 550 nm. Emission spectra of MOF, MCP-Me, MCP-Et, and MCP-iPr from 450 to 700 nm were collected after excitation at 400 nm.

Solution stability: Stability was determined using a modified previous reported procedure.[2] To summarize, 1 μM MOF, MCP-Me, MCP-Et, and MCP-iPr were incubated at 37°C in 1X PBS (Fisher, Waltham, MA) and at 37°C in an imaging solution (Fisher Gibco Fluorobrite DMEM supplemented with 20 mM HEPES at pH 7.4) for 3 h with the fluorescence intensity (λ_{ex}=460 nm, bandwidth=20 nm; λ_{ex}=550 nm, bandwidth=50 nm) recorded every 1 min in triplicate.

Solution stability at Variable pH: Stability at variable pH was determined using a modified previous reported procedure.[2] To summarize, 1 μM MOF, MCP-Me, MCP-Et, and MCP-iPr with 0.1% DMSO were prepared in 20mM glycine (pH 2.0-3.0),[2,51] acetate (pH 4.0-5.0),[2,51] phosphate (pH 6.0-8.0),[2,52] Tris (pH 9.0),[2,52] or CAPS (pH 10.0),[2,51] buffer and incubated for 30 min at 37°C. After incubation, the fluorescence intensity (λ_{ex}=460 nm, bandwidth=20 nm; λ_{ex}=550 nm, bandwidth=50 nm) was recorded in triplicate.

Results

First, I determined the fluorescent properties of the MCP series by measuring the fluorescence spectra of each probe in relation to its shared core fluorophore, 3-O-Methylfluoroscein (MOF). MOF has an excitation bandwidth centered at 460 nm and an emission bandwidth focused at 515 nm (Figures 4-6). As expected, each probe (MCP-Me, MCP-Et, and MCP-iPr) demonstrated very low relative fluorescent intensity (RFI) in comparison to MOF (Figures 4-6). Next, I sought to evaluate the stability of the MCP series in solution over time. I performed this assessment in two different solutions, PBS and the imaging solution we use for microscopy studies. First, I incubated MCP-Me, MCP-Et, and MCP-iPr at 37 °C at pH 7.4 in 1X PBS buffer for 3 hours to observe the stability of each probe over time (Figure 7). In the first hour, each probe produced no fluorescence, and after three hours, less than 10% fluorescence was recorded for each probe. Next, I incubated MCP-Me, MCP-Et, and MCP-iPr at 37°C in imaging solution for 3 hours and measured changes in fluorescence over time (Figure 8). In comparison to PBS incubation there was an increased fluorescent response in this solution, with the most significant change produced in MCP-Me. MCP-Et was found to be stable for at least one hour and MCP-iPr was stable for all three hours. Finally, I evaluated the stability of each probe across various pH levels (Figure 9). I found MCP-Et and MCP-iPr to be stable across all pH tested (pH 2.0-10.0).

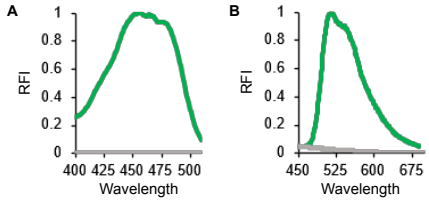

Figure 4. A. Excitation and **B.** emission fluorescence spectra of MCP-Me (gray) compared to MOF (green)

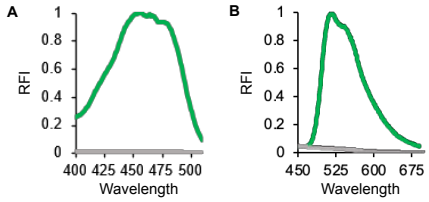

Figure 5. A. Excitation and **B.** emission fluorescence spectra of MCP-Et (gray) compared to MOF (green)

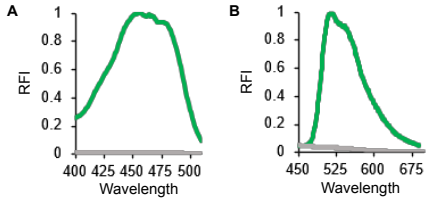

Figure 6. A. Excitation and **B.** emission fluorescence spectra of MCP-iPr (gray) compared to MOF (green)

Discussion

In this study, I first evaluated the emission and excitation of our probes in comparison to their structural fluorophore to determine if the probes possessed natural fluorescence. The absence of fluorescence demonstrated by MCP-Me, MCP-Et, and MCP-iPr indicates that the probes are not fluorescent on their own and must be hydrolyzed to produce fluorescence (Figures 4-6).

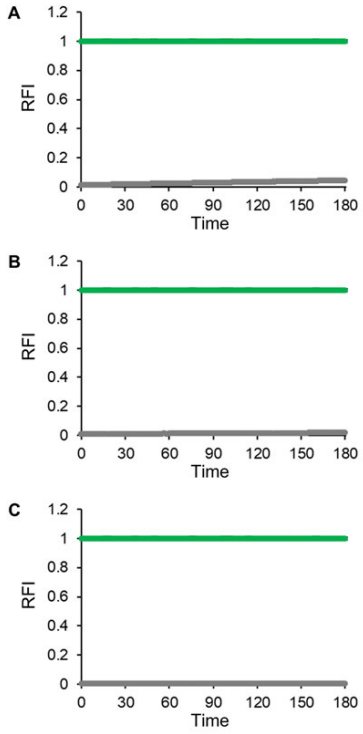

Figure 7. Solution Stability of **A.** MCP-Me, **B.** MCP-Et, or **C.** MCP-iPr (gray) compared to MOF (green) at room temperature at pH 7.4 in 1X PBS buffer

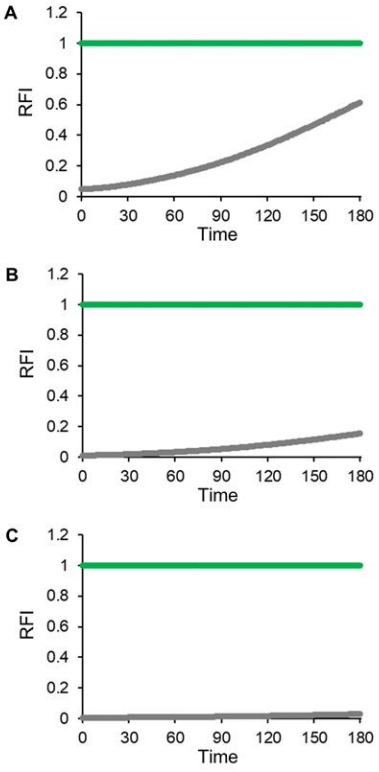

Figure 8. Solution Stability of **A.** MCP-Me, **B.** MCP-Et, or **C.** MCP-iPr (gray) compared to MOF (green) at 37°C at pH 7.4 in 20mM Fluorobrite DMEM HEPES

In solution stability studies of PBS, I observe that each probe maintains stability and produces no fluorescence in the first hour and less than 10% fluorescence after 3 hours. This suggests that MCP-Me, MCP-Et, and MCP-iPr do not hydrolyze to MOF in the absence of CESs and therefore are stable in PBS. In comparison to PBS, stability studies in imaging solution demonstrated greater instability for each probe. This can be attributed to the imaging solution being a more complex environment due to the presence of amino acids in DMEM that could hydrolyze MCP-Me and MCP-Et in the absence of CESs. While I see a greater hydrolysis in the absence of CESs, MCP-Et and MCP-iPr are stable for at least an hour. This indicates that MCP-Et and MCP-iPr can be used in live cell imaging experiments where we limit the experiment to under an hour;[2] however, it also suggests that we can further optimize our design to increase stability. Lastly, I evaluated the stability of each probe across the pH range of 2.0-10.0. I found

that MCP-Me produced little fluorescence through pH 4.0, but then began to produce fluorescence from pH 5.0-10.0. Although less than 20% hydrolysis is observed, this compromises MCP-Me's utility for live cells. MCP-Et and MCP-iPr produced no fluorescence from pH 2.0 to 8.0 and produced less than 20% fluorescence from pH 9.0-10.0. In comparison to FCP-1, our previously developed probe with the same carbonate group, all probes offer a higher level of stability. FCP-1 produced more than 40% fluorescence at pH 10.0, while MCP-Me and MCP-iPr produced less than 20%.[2] Given FCP-1's suitability in live cells, I can also conclude that MCP-Et and MCP-iPr would be suitable for use in live cells. In addition to being suitable, these data also suggest that MOF-based probes are more stable in basic environments than fluorescein-based probes.

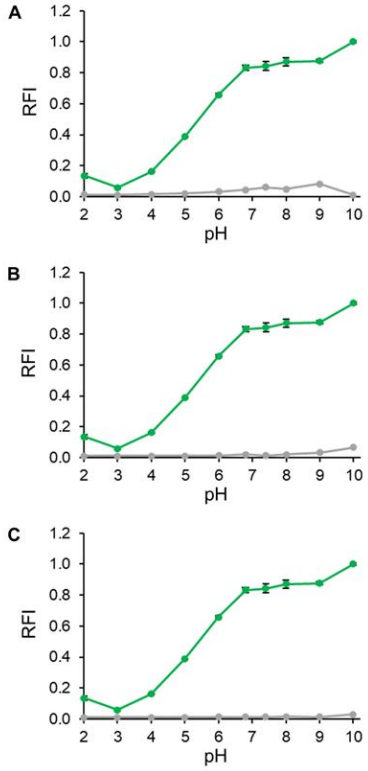

Figure 9. Solution stability of **A.** MCP-Me **B.** MCP-Et **C.** MCP-iPr (gray) compared to MOF (green) at variable pH (2.0-10.0)

Conclusions

In summary, MCP-Et and MCP-iPr are stable under *in vitro* conditions that mimic the conditions for live cells. MCP-Me, while unstable in imaging solution, could potentially still be useful in studying CES activity *in vitro* under certain conditions, but is unsuitable for use in live cells. In the future, I plan to characterize the Michaelis-Menten kinetics of the MCP series interaction with CESs and eventually deploy them for use in live cells. Overall, MCP-Et and MCP-iPr are suitable fluorescent probes for CES1 study *in vitro* and show promise for utility in live cells. By providing better tools to study CES1 activity, we can increase the efficacy of clinical therapies by assessing the patient's ability to metabolize CES1 mediated drugs prior to prescription. This will ensure that the patient is being treated with pharmaceuticals that are suitable with their body's chemistry, therefore increasing successful patient outcomes.

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