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Identification of Tissue-Restricted Bioreaction Suitable for in Vivo Targeting by Fluorescent Substrate Library-Based Enzyme Discovery

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Supporting Information

ABSTRACT: Tissue-restricted bioreactions can be utilized to design chemical-biological tools and prodrugs. We have developed a fluorescent-substrate-library-based enzyme discovery approach to screen tissue extracts for enzymatic activities of interest. Assay-positive candidate proteins were identified by diced electrophoresis gel assay followed by peptide mass fingerprinting. We discovered that pyruvyl anilide is specifically hydrolyzed by carboxylesterase 2 (CES2), which is predominantly localized in the liver and kidney. We show that the pyruvyl targeting group/CES2 enzyme pair can be used to deliver the 7-amino-4-methylcoumarin fluorophore specifically to the liver and kidney in vivo. Our screening approach should be useful to find other masking group/enzyme pairs suitable for development of fluorescent substrates and prodrugs.

One of the aims of chemical biology is to develop chemical tools that enable researchers to visualize or modulate cellular functions in a spatiotemporally controlled manner by utilizing specific bioreactions.[1] For example, a bioreaction commonly used for tool development is cleavage of the β-galactopyranosyl group by β-galactosidase.[2] However, in view of the enormous number of potentially targetable enzymes in the proteome,[1c,3] the currently available choice of targeting moiety/target enzyme pairs is extremely limited. In particular, identification of selective bioreactions catalyzed by endogenous enzymes with restricted tissue- or cell-type distribution is urgently required to provide a basis for tissue- or cell-type-specific delivery of imaging agents or functional molecules in the human body without the need for gene manipulation. Therefore, the aim of the work described here was to discover bioreactions (targeting moiety/target enzyme pairs) with potential applicability for the development of functional molecules. The search for suitable reactions, especially efficient ones, is not straightforward; it is not enough simply to compare protein expression levels in different tissues, since enzymatic activity is not necessarily directly related to the protein expression level,[1c,4] and the contribution of a particular enzyme to the metabolism of individual substrates cannot be easily estimated. Therefore, we employed a library of fluorescent substrate probes, which enables direct readout of the enzymatic activity with high sensitivity and high throughput,[5] and we searched for suitable activities by monitoring the susceptibility of the probes to enzymatic reactions in extracts of tissue samples. To our knowledge, such a strategy has never previously been tried for the present purpose. Characterization of enzymes mediating detected activities is potentially a bottleneck, but our laboratory has recently developed an efficient method, diced electrophoresis gel assay[6] followed by peptide mass fingerprinting, to characterize the target enzyme of a given fluorescent substrate in biological samples. Here this approach enabled us to discover that pyruvyl anilide is cleaved in the liver and kidney by carboxylesterase 2 (CES2) with high efficiency and high selectivity. To demonstrate its usefulness, we show that the pyruvyl moiety/CES2 pair can be used to deliver the 7-amino-4-methylcoumarin (AMC) fluorophore specifically to the targeted tissues.

Fluorescent probe libraries for aminopeptidases,[7] proteases,[8] and oxidoreductases[9] have been well-developed on the basis of existing biochemical knowledge, but in the present work we decided to focus on activities for hydrolysis of amide bonds involving biologically occurring carboxylic acids other than amino acids because they are applicable for the design of functional small molecules and prodrugs and because enzymes mediating these reactions have not been systematically studied and characterized to date. We prepared a library of amides based on the AMC fluorophore.[10] Protection of the 7-amino group quenches the fluorescence, and hydrolysis releases fluorescent AMC. Representative structures and chemical characteristics are shown in Scheme S1, Table S1, and Figure 1. Acetyl (**1**), propionyl (**2**), *n*-hexyl- (**3**), acryl- (**4**), benzoyl- (**5**), 2-nitrophenacyl- (**6**), 4-nitrobenzoyl- (**8**), diglyoxylyl- (**9**), and trifluoroacetyl-AMC (**14**) were prepared as derivatives of general small-molecule carboxylic acids. Methyloxalyl-AMC (**13**) was prepared as a representative of other nucleophiles. Formyl- (**7**), pyruvyl- (**10**), and nicotinyl-AMC (**12**) were prepared as controls for hydrolysis of biologically occurring formyl and pyruvyl amides and nicotinamides. The *N*,*N*-

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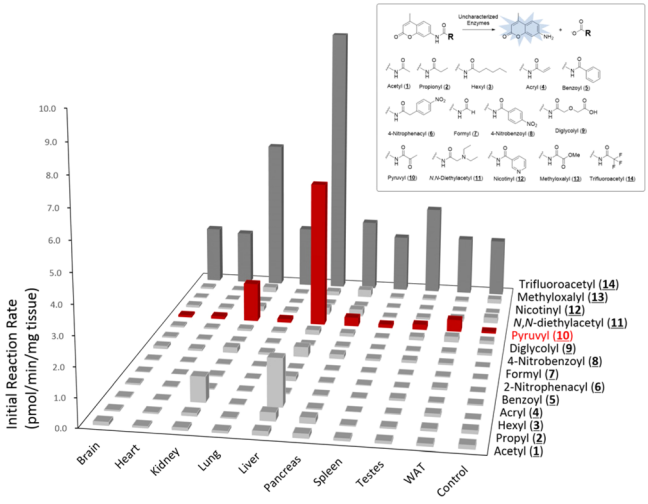

Figure 1. Fluorescence increase rates of fluorescent probes (10 μM) after incubation with mouse tissue lysates (1 mg/mL) in phosphate buffer (pH 7.4) containing $CaCl_2$ and $MgCl_2$.

diethylaminoacetyl group (**11**) was derived from the protective group of the bioactive drug lidocaine.

Fluorometric assays of these probes were performed in extracts of various mouse tissue lysates. Interestingly, we found that the pyruvyl amide-type probe (**10**, Pyr-AMC) was hydrolyzed with high specificity in liver and kidney lysates (Figure 1). There are several reports on protease probes with high tissue selectivity,[1c] but tissue-selective activity was rarely seen among the fluorescent substrates for aminopeptidases that we tested, such as Leu-AMC (for leucine aminopeptidase), Met-AMC (for methionine aminopeptidase), and Ala-AMC (for multiple targets) (Figure S1). Therefore, we found the case of Pyr-AMC interesting. It is known that N-pyruvylated peptides are generated by radical-mediated degradation of proteins (oxidative stress),[11] but to date no enzymes have been reported to exhibit metabolic activity toward pyruvyl amides. Thus, we used our recently developed diced electrophoresis gel assay[6] to identify the target enzyme of Pyr-AMC. After optimizing the separation conditions of native electrophoresis, we were able to detect two protein spots that exhibited hydrolytic activity toward Pyr-AMC (Figures 2A,B and S2). Each spot likely contains multiple proteins, and we used peptide-mass-fingerprinting-based characterization to generate a list of candidate target proteins, as summarized in Table S2. Proteases such as cathepsin B and trypsin, which we expected to have potential amide-cleaving activity, did not show hydrolytic activity toward Pyr-AMC (Figure S3). Certain carboxylesterases (CESs) appeared in both spots, and we focused on this class of enzymes for further investigation since some CESs are known to hydrolyze amides as well as esters.[12] Indeed, the spots coincided with activity toward fluorescein dibutyrate (FDBu), a general esterase probe[6] (Figure 2C), and the general CES inhibitor bis(4-nitrophenyl) phosphate (BNPP) was confirmed to block the fluorescence increase of Pyr-AMC (Figure S4). In order to further evaluate hydrolysis of Pyr-AMC by CESs, we next prepared lysates of HeLa cells transiently expressing various mouse CESs (Figure S5). Among 19 CES subtypes in mouse,[13] we selected eight that are known to be present in the liver (including members of all three major subclasses, CES1, -2, and -3) and transfected their complementary DNAs into HeLa cells.

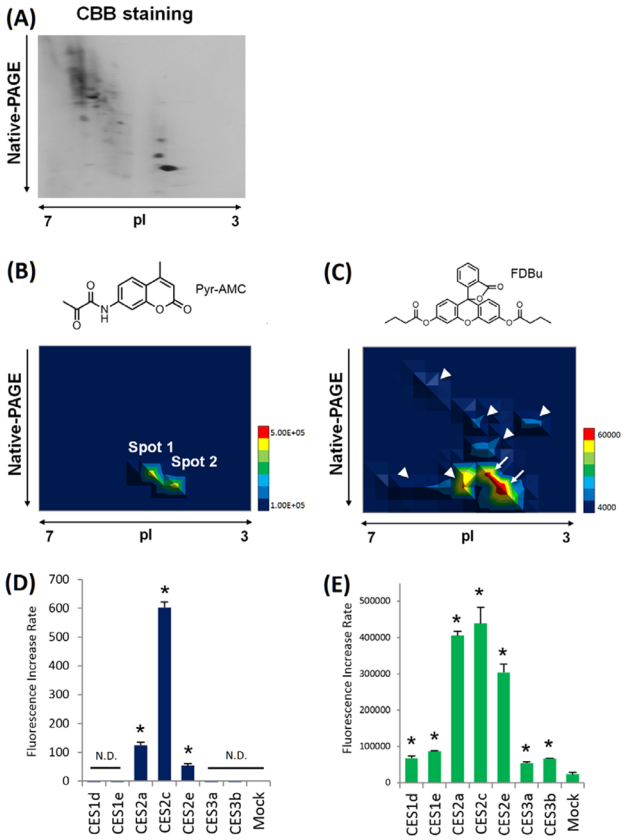

Figure 2. Characterization of CES2 as a pyruvyl anilide-hydrolyzing enzyme. (A) CBB-staining image of a two-dimensional electrophoresis gel of mouse liver lysate. (B, C) Results of two-dimensional diced electrophoresis gel assay of mouse liver lysate with (B) Pyr-AMC (10 μM) or (C) FDBu (10 μM). Spots with white arrows in (C) correspond to the spots detected in (B), while spots with white arrowheads were not detected in (B). (D, E) Results of fluorometric assays of (D) Pyr-AMC and (E) FDBu with HeLa cell lysate expressing CES1, -2, and -3 isoforms (n = 3). * denotes significantly increased activity compared with mock-transfected cells ($P < 0.05$).

Expression of the desired proteins was confirmed by the observation of increased hydrolytic activity toward FDBu in the lysates. We confirmed that fluorescence activation of Pyr-AMC occurred only in lysates of CES2-expressing cells (Figure 2D). Comparison of the activities with those in liver lysate by means of one-dimensional and two-dimensional diced electrophoresis gel assay (Figures S6 and S7) as well as the results of Western blotting confirmed that the major target of Pyr-AMC is CES2. It is noteworthy that whereas FDBu showed elevated activity with all classes of esterases (Figure 2E), Pyr-AMC showed elevated activity only in CES2-expressing lysates; in other words, Pyr-AMC showed complete selectivity for CES2 among various CESs. We studied the substrate scope of various pyruvyl anilide-bearing molecules for CES2 and found that pyruvyl anilides with fluorophores other than AMC were also good substrates of CES2 with comparable selectivities (Figure S8), whereas pyruvyl amides with aliphatic amines (such as naturally occurring pyruvyl peptides) were not (Figure S9).

Therefore, the pyruvyl anilide structure seems to be necessary and sufficient for targeting of CES2. Carboxylesterases are involved in metabolism of exogenous compounds[14] and lipid metabolism,[15] but their physiological roles are not fully understood. However, CES2 levels are altered in various

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pathological states,[16] and some tumor cells express elevated levels of CES2.[17] After confirming the selectivity of Pyr-AMC for CES2 over CES1 in the case of human carboxylesterases (Figure S10), we tested whether Pyr-AMC can be used to detect CES2 activity at the cellular level. We prepared lysates of seven cancer cell lines and studied the correlation between the CES2 expression level and the fluorescence increase of Pyr-AMC. We confirmed that marked fluorescence was generated by lysates of HepG2 and SKOV3, cell lines that are reported to express high levels of CES2[17] (Figure 3A). Although we cannot

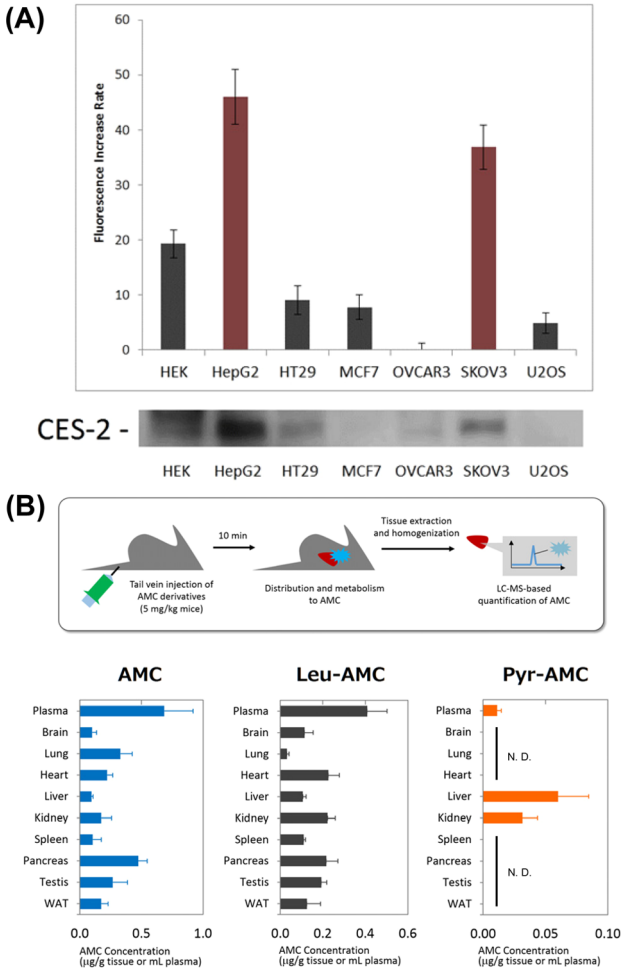

Figure 3. CES2-mediated hydrolysis of pyruvyl anilides for in vivo targeting of functional molecules. (A) Fluorescence intensities of Pyr-AMC (1 μM) in lysates (0.1 mg/mL) of various cells. (B) Tissue distribution of 7-amino-4-methylcoumarin (AMC) 10 min after intravenous injection of AMC, Leu-AMC, or Pyr-AMC (5 mg/kg) into mice (n = 3).

completely exclude a possible contribution of differential expression of hydrolytic enzymes other than CES2 to the fluorescence increases, these results support the idea that Pyr-AMC can detect endogenous activity of CES2 with sufficient sensitivity and selectivity for practical applications.

Finally, we examined the availability of this bioreaction for in vivo delivery of functional molecules. After intravenous injection of Pyr-AMC into mice, the formation of fluorescent AMC was observed exclusively in the liver and kidney, where CES2 expression levels are high (Figure 3B). A low concentration of AMC was also detected in plasma, but since no Pyr-AMC hydrolytic activity was detected in mouse serum (Figure S11), this might be due to leakage of AMC from the above two tissues.

Tissue-selective activation of functional molecules for imaging purposes has already been achieved by targeting β-galactosidase as a reporter enzyme,[18] but here we have succeeded in tissue-selective activation by utilizing an endogenous, tissue-restricted enzyme without the need for any genetic modification. These results are in sharp contrast to the results of in vivo targeting of AMC with a leucyl group (Leu-AMC), which proved unsuccessful; AMC was detected in all of the tissues tested, and the highest concentration was observed in plasma. This is probably due to the ubiquitous presence of the targeted enzyme (presumably leucine aminopeptidases). We also synthesized a pyruvyl-amidated methyl ester derivative of p-aminosalicylic acid (PAS), an NF-κB inhibitor that has also been used to treat tuberculosis, and confirmed that the prodrug, PAS methyl ester, was released in the presence of CES2 (Figure S12). This result suggests that the discovered bioreaction would be available for delivery of druglike molecules. Several commercial prodrugs are activated by CESs (e.g., capecitabine[17] and iriniotecan[19]), but they are metabolized by both CES1 and CES2 in the human body. Our reaction was highly selective for CES2 in both mouse and human. We think that it led to the stability of compounds in blood and nontargeted tissues where CES2 was not present (Figure S11) and should enable successful utilization of this reaction for tissue-selective delivery of drugs by intravascular injection; a sufficiently high reaction rate might also be a contributory factor (Figure S13). It should be noted that CESs are also present in digestive tissues, such as the stomach and small and large intestines,[20] so the possibility that activities in those tissues contribute to the metabolism of Pyr-AMC should be considered, especially in the case of oral administration. In any event, we believe that our screening system will also be applicable for the discovery of other novel enzyme–substrate pairs suitable for targeting other tissues. Studies along the line are already in progress.

In conclusion, we have utilized a fluorescent-substrate-library-based enzyme discovery approach to identify a novel tissue-restricted bioreaction, i.e., cleavage of pyruvyl anilide by CES2 in the liver and kidney. We showed that the pyruvyl targeting group/CES2 enzyme pair can be used to deliver the AMC fluorophore specifically to the liver and kidney in vivo. We also confirmed that CES2 hydrolyzes pyruvyl-amidated PAS methyl ester, indicating that other functional molecules should be deliverable with this system. This work illustrates the ability of the target discovery approach to identify novel bioreactions suitable as a basis for the future development of a range of chemical-biological tools, such as fluorescent substrates and prodrugs. We believe that this approach will be broadly useful to characterize novel protein functions in the enzymome (the complete set of enzymes active in a cell)[21] and also to identify characteristic target candidates in various pathological states.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b05884.

Methods and supporting schemes, tables, and figures (PDF)

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Notes

The authors declare no competing financial interest.

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