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A new visual screening assay for catalytic antibodies with retro-aldol retro-Michael activity

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Abstract—Fast and convenient methods are required for the detection of novel catalysts. We have developed a new assay to allow direct visualization of retro-aldol retro-Michael catalytic activity and have demonstrated it with catalytic antibody 38C2. The assay is based on a catalytic cleavage of a physiologically stable substrate to release 3,4-cyclohexeneoesculetin. The latter then reacts with iron(III) to generate a non-soluble complex that precipitates in the form of a black dye. This assay may be used for screening new catalysts for retro-aldol retro-Michael activity with improved efficiency for specific prodrug activation.

The tandem retro-aldol retro-Michael reaction catalyzed by antibody 38C2 is an efficient cleavage reaction that has potential for prodrug activation.[1–3] This reaction is not known to be catalyzed by natural enzymes and, therefore, non-specific prodrug activation by endogenous enzymes should not occur. The unique activity of this antibody is derived from an ε-amino-lysine residue with a significantly perturbed p*K*a (5.8) buried deeply in the antigen binding pocket. This lysine is capable of reacting with a ketone functionality at physiological pH and consequently generates a highly reactive enamine species.[4] Several variations of fluorogenic assays have been developed to monitor aldolase activity[5,6], including a direct visual detection assay[7] and one amperometric assay.[8] Here, we report on a new visual detection assay for the screening of retro-aldol retro-Michael catalytic activity.

Simple, sensitive in vitro detection of a catalyst can be achieved if the desired reaction converts a non-visible substrate into a new product that can react with an additional reagent to generate a colorful precipitate. Compound **1,** generated from 3,4-cyclohexenoesculetin **2** and a retro-aldol retro-Michael linker, is a promising example of such a substrate (Fig. 1). Antibody 38C2 catalyzes the retro-aldol retro-Michael cleavage reaction to generate an amine intermediate that is cyclized spontaneously to release 3,4-cyclohexenoesculetin. In the presence of iron(III) ion, 3,4-cyclohexenoesculetin forms complex **3**, a black precipitate that is constructed of three molecules of 3,4-cyclohexenoesculetin per ion of iron III (Fig. 2). This assay has been recently used for staining cells with cloned DNA that contains the sequence for β-galactosidase.[9] We sought to replace the β-galactosidase substrate with a retro-aldol retro-Michael substrate in order to detect the aldolase activity of catalytic antibody 38C2.

Substrate **1** was synthesized as outlined in Figure 3.[10] 3,4-Cyclohexenoesculetin **2** was selectively protected as methoxymethyl-ether **4** and then reacted with 4-nitrophenyl-chloroformate to give the 4-nitrophenyl-carbonate **5**. Reaction of the retro-aldol retro-Michael linker **6a** (prepared as previously described[1]) with carbonate **5** afforded compound **6**, which was deprotected with TFA to give substrate **1**.

To determine whether compound **1** is stable under physiological conditions, it was incubated in phosphate-buffered saline, pH 7.4 (PBS) at 37 °C for 72 h. No decomposition was observed. The reaction of substrate **1** upon incubation with catalytic antibody 38C2 was monitored by reverse-phase HPLC. We found that the antibody indeed catalyzed the retro-aldol retro-Michael

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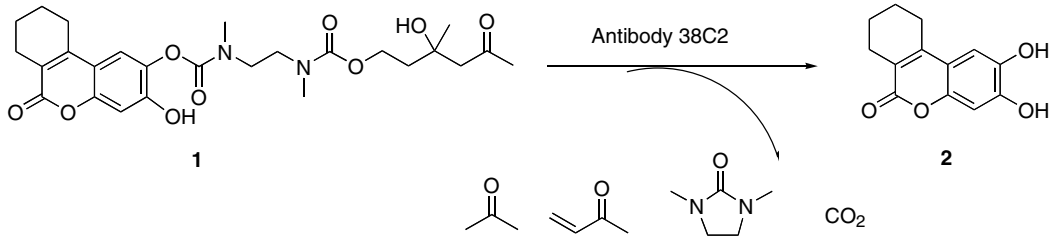

Figure 1. Antibody 38C2 catalyzed retro-aldol retro-Michael cleavage reaction of substrate **1**, followed by spontaneous cyclization to release 3,4-cyclohexenoesculetin **2**.

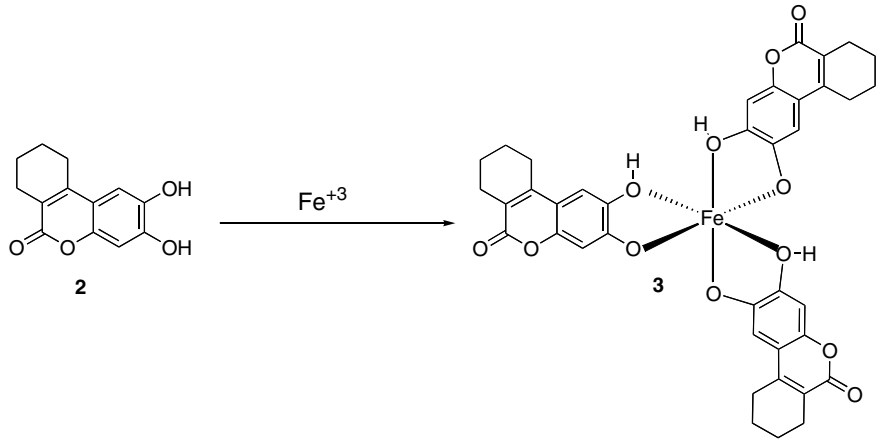

Figure 2. Three molecules of 3,4-cyclohexenoesculetin react with iron(III) ion to generate a complex that forms a black precipitate.

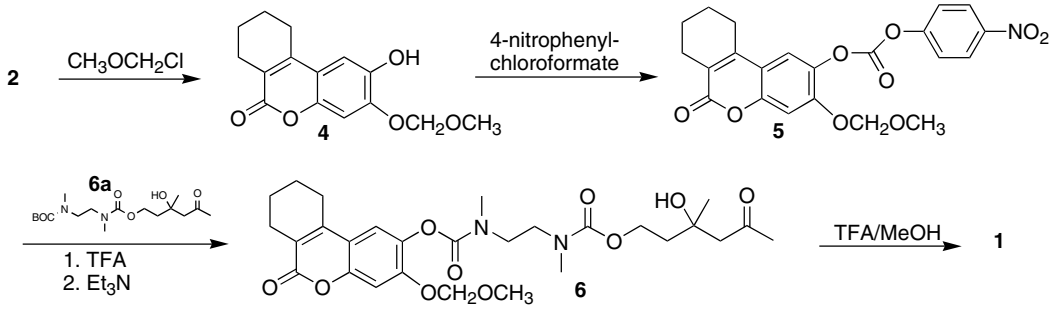

Figure 3. Chemical synthesis of substrate **1**.

cleavage reactions to generate 3,4-cyclohexenoesculetin. As shown in Figure 4, compound **1** was gradually converted to the product 3,4-cyclohexenoesculetin; no amine intermediate was observed. Lack of detection of the intermediate can be explained by the fast cyclization step that occurs spontaneously after the retro-aldol retro-Michael cleavage. Since substrate **1** was synthesized in its racemic form, one enantiomer was cleaved much faster by the catalytic antibody than the other. This results in a slower reaction rate after 50% conversion of the substrate to product.

To evaluate the utility of the substrate in a visual assay, we incubated substrate **1** and iron(III) chloride with a catalytic amount of antibody 38C2 in PBS (pH 7.4). A control reaction included substrate **1** and iron(III) chloride in PBS (pH 7.4). A generation of a black precipitate was clearly observed in the tube with catalytic antibody

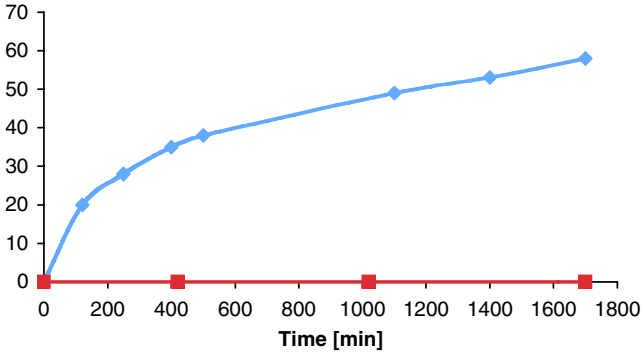

Figure 4. Conversion of substrate **1** to 3,4-cyclohexenoesculetin versus time. Substrate **1** [500 μM] in PBS (pH 7.4) with catalytic antibody 38C2 (50 μM) at 37 °C (blue). Substrate **1** in the absence of the antibody (red).

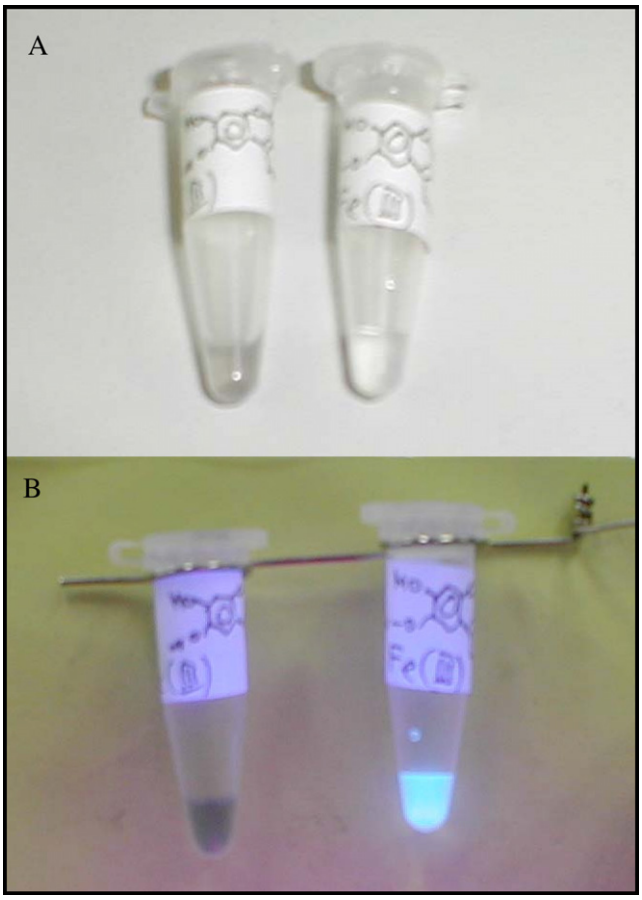

Figure 5. (A) Photograph of a tube containing substrate **1** (500 μM), iron(III) chloride (200 μM), and catalytic antibody 38C2 (50 μM) (on the left), and a tube with substrate **1** and iron(III) chloride (on the right). (B) Photograph of the tubes described in (A) under 340 nm UV light.

38C2, while the control reaction remained completely clear (Fig. 5A). 3,4-Cyclohexenoesculetin and derivative **1** are both fluorescent. When the control reaction was exposed to 340 nm UV light, fluorescence was clearly observed; no fluorescence was observed in the tube containing antibody (Fig. 5B). This phenomenon is explained by the reaction of the 3,4-cyclohexenoesculetin with iron(III) ion, resulting in metal complex **3** that quenches the fluorescence generated by the free 3,4-cyclohexenoesculetin. We evaluated the sensitivity of the assay by analyzing a series of reactions prepared with a range of antibody concentrations. The black precipitate and quenched UV signal could be detected down to 1 μM of catalytic antibody 38C2.

One important advantage of this assay is that it may be applied for selection of proteins expressed from cloned DNA in *Escherichia coli* colonies. If the expressed protein has a retro-aldol retro-Michael catalytic activity, it will form the black dye/iron complex that will precipitate. Since the black dye is not water-soluble, it will gradually accumulate in the cell and form a visual stain that will indicate the colony that expresses a protein with the desired catalytic activity.

In summary, we have developed a new screening assay for retro-aldol retro-Michael catalytic activity that can be clearly visualized. The assay is based on a catalytic cleavage of a physiologically stable substrate to release 3,4-cyclohexenoesculetin. The substrate is cleaved by catalytic antibody 38C2. 3,4-Cyclohexenoesculetin reacts with iron(III) ion to generate a non-soluble complex that precipitates as a black dye. The black dye was clearly observed in the solution in the presence of the antibody, whereas the control solution remained clear. This assay may be used in a search for new catalysts with retro-aldol retro-Michael activity that can have improved efficiency for specific prodrug activation.

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10. Compound **4**. The commercially available 3,4-cyclohexenoesculetin **2** (100 mg, 0.431 mmol) and *tert*-butyl potassium hydroxide (48.36 mg, 0.431 mmol) were dissolved in 2 mL DMF and cooled to 0 °C. Chloromethyl-methyl-ether (33 μL, 0.431 mmol) was added dropwise to the stirred solution. The reaction mixture was stirred for 2 h at room temperature and monitored by TLC (EtOAc/He, 3:1). After completion, the mixture was diluted with EtOAc, washed with satd solution of NH_4Cl, dried over sodium sulfate, and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel (EtOAc/Hex, 2:3) to give compound **4** (67 mg, 56%). ^1H NMR (200 MHz, $CDCl_3$): δ = 7.09 ppm (2H, s); 3.53 (2H, s); 2.72 (2H, m); 1.86–1.81 (4H, m); 1.58 (3H, s).
Compound **5**. Compound **4** (67 mg, 0.24 mmol) was dissolved in dried 2 mL THF. Triethylamine (30 μL) was added. The reaction mixture was cooled to 0 °C and 4-nitrophenylchloroformate (48.7 mg, 0.24 mmol) dissolved in 2 mL THF was added dropwise. The reaction mixture was stirred at room temperature for 1 h and monitored by

TLC (EtOAc/He, 3:1). After completion of reaction, the precipitate was recovered by filtration and the remaining solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel (EtOAc/Hex, 2:3) to give compound **5** in the form of white powder (69.5 mg, 65%). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 8.3 ppm (2H, d, J=7); 7.5 (2H, d, J=7); 7.4 (1H, s); 7.23 (1H, s); 3.5 (2H, s); 2.72 (2H, m); 2.58 (2H, m); 1.84 (4H, m) 1.56 (3H, s).

Compound **6**. Retro-aldol–retro-Michael linker **6a** (57.1 mg, 0.156 mmol) was deprotected from the Boc with 1 mL TFA for 2 min at 0 °C. The excess of the acid was removed under reduced pressure and the amine salt was dissolved in 2 mL DMF. Compound **5** (69.5 mg, 0.156 mmol) was added in with 0.5 mL of triethylamine and the solution was stirred for 10 min. The reaction was monitored by TLC (EtOAc/MeOH, 9:1). After completion, the DMF was removed under reduced pressure and the crude product was purified by flash chromatography (MeOH/EtOAc, 2:98) to give pure compound **6** in the form of white powder (47 mg, 54%). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.14 (2H, s); 4.24(2H, m); 3.51–3.48 (4H, m); 3.13 (2H, s); 3.04 (2H, s); 2.99–2.98 (3H, m); 2.72 (2H, m); 2.65 (2H, d, J=4); 2.58 (2H, m); 2.17 (3H, s); 1.88 (2H, m); 1.84 (4H, m); 1.23 (3H, s). MS(FAB): $C_{28}H_{38}N_2O_{10}$ $[M+Na]^+$ 585.1

Compound **1**. Compound **6** (47 mg, 0.08 mmol) was dissolved in 1 mL DCM and 1 mL TFA at 0 °C. The solution was stirred for10 min and reaction was monitored by TLC (EtOAc/MeOH, 9:1). After completion, the solvent was removed under reduced pressure and purified by column chromatography on silica gel (EtOAc, 100%) to give desired compound **6** (30 mg, 75%). MS(FAB): $C_{26}H_{34}N_2O_9$ $[M+H]^+$ 519.1.