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On-Chip Fragment-Based Approach for Discovery of High-Affinity Bivalent Inhibitors

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Traditional lead-discovery approaches often rely on synthesizing and screening large numbers of compounds to identify a few inhibitor molecules (Figure 1 A). But even increasingly large compound collections have not directly improved the number of discovered lead compounds. Structure–activity relationships (SAR) by using nuclear magnetic resonance spectroscopy (NMR) was developed in 1996 and initiated the so-called initial "fragment-based approach" (Figure 1 B).[1a] The method "fragment-based design by using NMR spectroscopy" provides a new opportunity to create synthetic molecules that regulate biological processes in cells with a specific affinity for a target protein.[1a–e] In addition to this, several technologies, such as X-ray crystallography[2a–c] and mass spectrometry,[2d,e] have been used to demonstrate fragment-based ligand discovery. Oligonucleotide-coded compound libraries,[2f] scaffold-based drug design,[2g] and phage display technology[2h] for ligand discovery have been reported recently. These fragment-based approaches have become a paradigm in the inhibitor discovery of proteins of interest for pharmaceutical companies and also for academic laboratories.

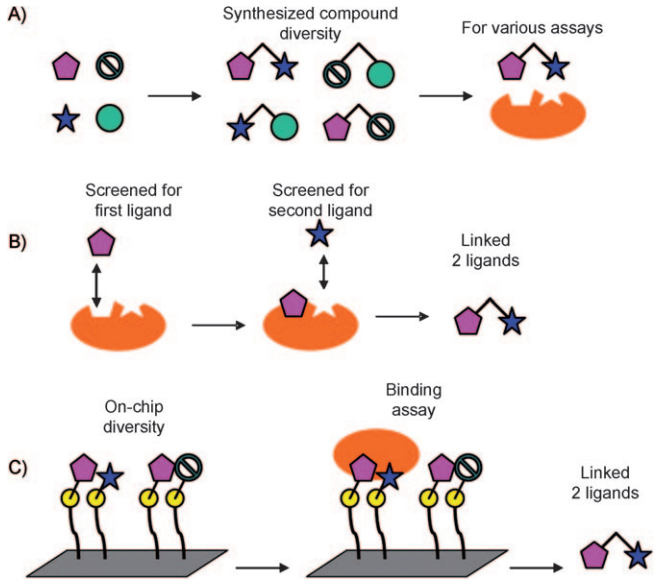

Figure 1. An outline of generating inhibitors for target proteins. A) Traditional combinatorial chemistry and high-throughput screening. B) Fragment-based ligand discovery with SAR by using NMR spectroscopy. C) Fragment combination array (FCA), described in this report.

However, current methods have several limitations, because many of these approaches require relatively large quantities of purified protein.[1e,3a,b] Additionally, many techniques also require the labeling of small molecules or proteins to identify the interaction. In many cases, it takes considerable effort to modify those molecules without loss of biological activity. With these issues in mind, we looked for a rapid approach for the discovery of fragments to generate high-affinity ligands of proteins.

We present initial studies of "fragment-based approaches" to binding assays by using a chemical array format, which we call a fragment combination array (FCA). In general, the "fragments" used in fragment-based approaches have low molecular weights or low affinities for the target protein.[3,4] Although chemical array technologies have been proposed to find useful binders for proteins in a high-throughput manner, it is difficult to detect the weak binding of proteins to immobilized "fragments" because of the necessary wash steps.[5] Thus, FCA involves a pair of these fragments immobilized on a glass slide at a proximate area through photogenerated carbene species (Figure 1 C). The chemical array experiments used in FCAs do not require the compounds to have a specific functional group,[6a–c] and tedious steps for protein purification are avoided.[7a–b]

To test the proof-of-concept of FCAs, we synthesized **1** and a variety of benzanilide derivatives **2a**–**2d** (Figure 2 A). A pipecolinic acid derivative (**1**) has been identified as a ligand for FKBP12 with an affinity in the micromolar range, and benzanilide derivatives have been obtained as binders that interact with the protein at a nearby binding site of **1**.[1a] A compound with nanomolar affinity for FKBP12 was discovered by tethering structures **1** and **2a**. Thus **1** and **2a**, if immobilized in close proximity on a slide surface, could be used to bind to FKBP12; an increase in binding signal at the mixed spot would be observed compared with areas spotted by individual fragments. To confirm this hypothesis, DMSO solutions of **1** and **2a** were premixed and spotted on the array slide.[6b] Solutions of **1** and **2a** also were spotted individually on the same slide. After incubation of cell lysates that overexpress RFP-fused FKBP12, an area that was spotted with **1** and **2a** exhibited significantly increased fluorescence compared with other areas where the compounds were immobilized individually (Figure 2 B). Although fluorescence was observed on the spot where **1** alone was immobilized, no significant fluorescence was obtained on the spot immobilized by **2a** alone relative to the background level.

With a higher concentration of individual compounds against a mixed solution, the pattern of signal intensities was

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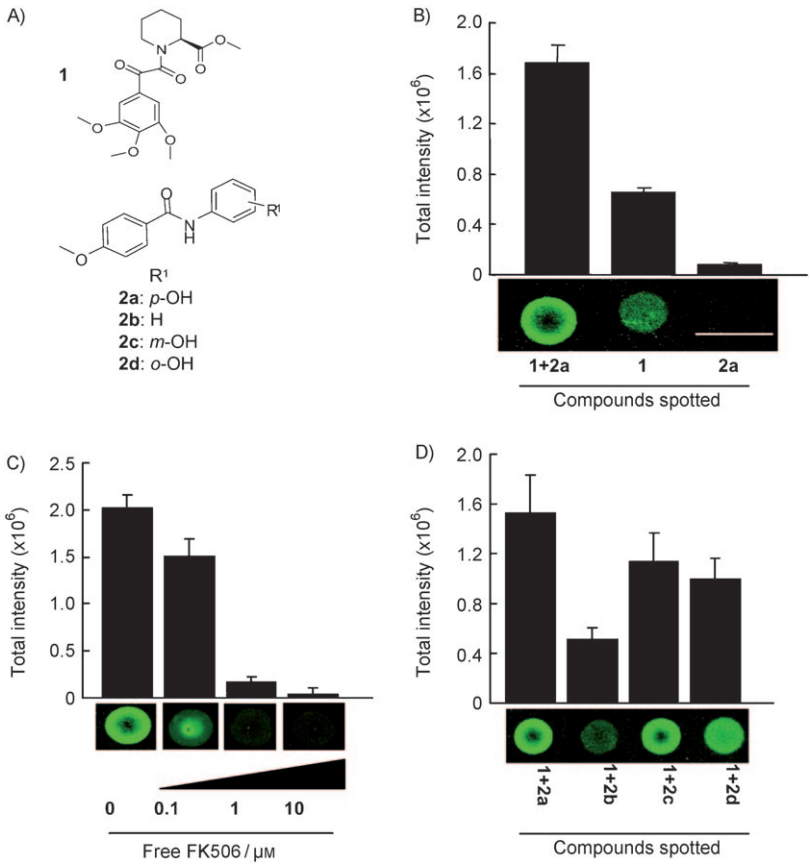


Figure 2. Detecting fluorescence signals in FCA. All slides were incubated with HEK293T cell lysates that overexpressed RFP-fused FKBP12 (3–4 mg mL^{-1}). The washed slide was scanned for fluorescence at 532 nm. The total intensity was corrected for background intensity. The error bars denote standard deviation over three replicates. A) Structure of binders to FKBP12. B) Solutions of mixed **1** and **2a** (each compound 10 mM) and of independent **2a** (10 mM) and **1** (10 mM) were printed on a photoaffinity-linker-coated slide. An image of the chemical array scanned for fluorescence is shown; white bar, 800 μm. C) A solution of mixed **1** and **2a** was arrayed, and the slide was incubated with the cell lysate in the presence of free FK506 with various concentrations (0.1–10 μM). D) Solutions of **1** mixed to **2a**, **2b**, **2c**, and **2d** were arrayed, and the slides were incubated with the cell lysates.

not changed (Figure S1 in the Supporting Information). These results show that one ligand increased the signal in cooperation with another one. Thus, **1** and **2a** were immobilized at appropriate densities in a spot on the slide, and this allowed their synergistic binding of FKBP12. The binding site for these fragments has been reported to be the same as that of FK506.[1a] The binding signal of the spot where **1** and **2a** were mixed and immobilized was blocked by dose-dependent competition of the addition of free FK506 (Figure 2C). The interaction of FKBP12 and mixed fragments that were immobilized by FCA was competed away by free FK506. Therefore, the binding between mixed fragments on FCA and protein is not an artifactual binding but a specific one. To determine whether substitution effects of mixed ligands could be observed on FCA, we used several benzanilide derivatives (**2a**–**2d**). The *para*-hydroxyl group on the aniline ring in **2a** was reported to contribute most for binding to FKBP12, although the *meta*-hydroxyl analogue **2c** retained similar binding;[1a] however, it was reported that an *ortho*-hydroxyl substitution **2d**, and the unsubstituted analogue **2b** had dramatically diminished binding activity due to a loss of the binding contribution of the hydroxyl group on the aniline ring. We found fluorescence signals on the slide with different strengths depending on the hydroxyl substitution position of **2** mixed with **1**(Figure 2D). Note that the spot where both **1** and **2a** were combined showed the strongest fluorescence signal relative to other pairs. On the other hand, the spot where **1** and **2b** were combined showed a very weak signal, which was almost the same as that of the spot immobilized by **1** alone (see, Figure 2B). The fluorescence intensities of spots, where **1**, and respectively, **2c** and **2d** were immobilized, were observed as the median of all spots. From these results, the SAR of these ligands to FKBP12 by FCA is found to be in good agreement with the SAR obtained by NMR spectroscopy. Further, the structural requirements for these ligands to be recognized by the protein were precisely determined by on-chip analysis.

The next goal was to examine whether FCAs could be applied to additional molecules. We chose carbonic anhydrase II (CAII) as a target protein because of the wealth of available information about the protein and its inhibitors.[8a–d] Therefore, aryl sulfonamide derivatives were first used as a scaffold for anchoring fragments to test several combinations of the pairs to generate novel high-affinity inhibitors. The anchor fragments were selected from a compound library, NPDepo (RIKEN Natural Product Depository),[9] according to three themes: 1) an aromatic sulfonamide property, 2) a low molecular weight ($\leq$350), and 3) a prediction for eventual follow-up synthesis. We selected five scaffolds that were constituted by four aromatic sulfonamide derivatives **A**–**C** and **E**, and an amide derivative **D**, which was selected as a negative control for our screen (Figure 3A). After that, a total of ten structurally diverse scaffolds, **F1**–**F10**, were selected as block fragments on the basis of their low molecular weight (MW $\leq$350), solubility (ClogP $<$3), and structural diversity, and they were employed as complementary fragments to the anchor fragments.

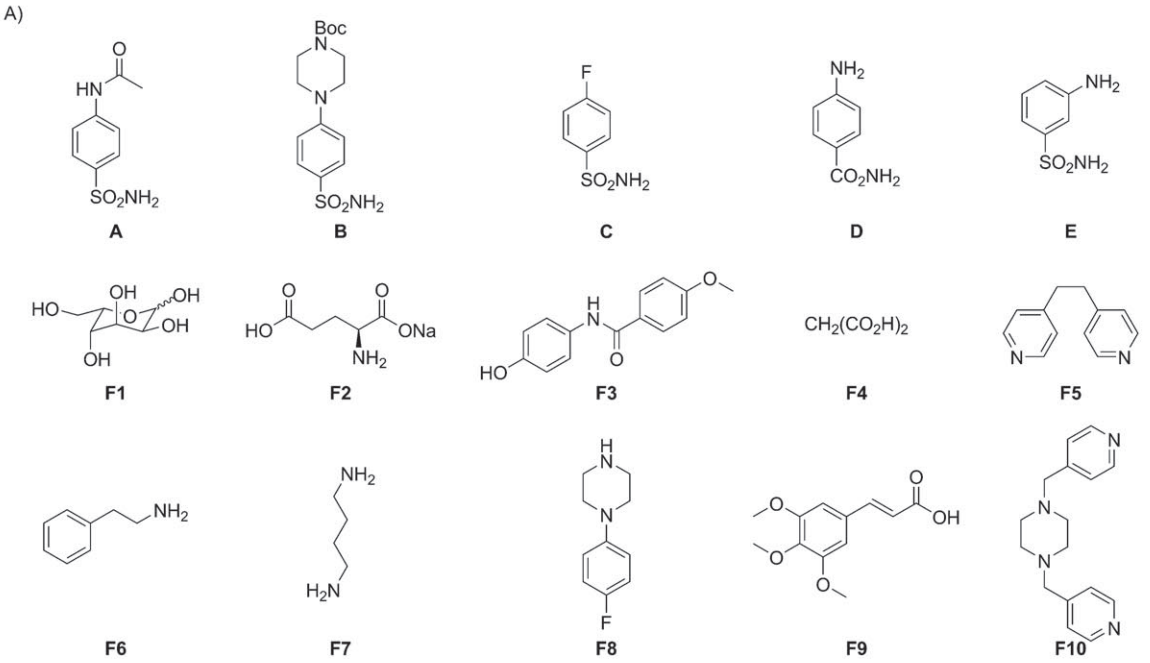
These results suggest that the structural motifs of these two fragments, an aromatic sulfonamide derivative (**C**) and a pyrimidyl piperazine derivative (**F10**), contribute to the binding to CAII. When ligands were immobilized on the array slide, a photogenerated carbene from a photoreactive linker was known

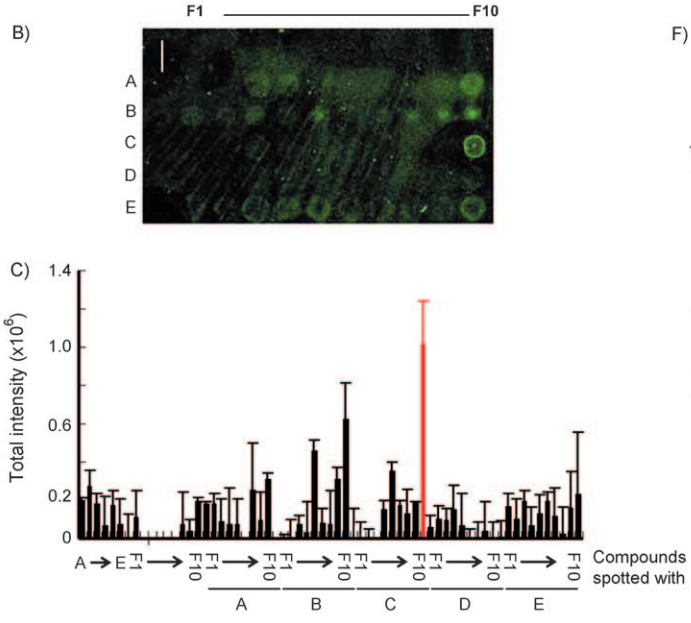
to insert C–H and O–H bonds into ligands at random.[6c] The structure of **F10**, with two pyridines and one dimethyl piperazine, is so symmetric that either of the two pyridines was expected to bind even though the other one was used for the immobilization. Additionally, some groups have reported that the *para* substitution of aromatic sulfonamide inhibitors enhanced the ability of the synthesized compounds to bind to CAII.[10a–c] Thus, we decided to place a pyridine moiety on the aromatic sulfonamide in the *para* position to yield a bivalent inhibitor design. Ideally, the linker should be as short as possible, so that the benefits of the fragment assembly can be received.[11,12] Thus, we chose the methyl piperazine moiety in **F10** as a short linker.

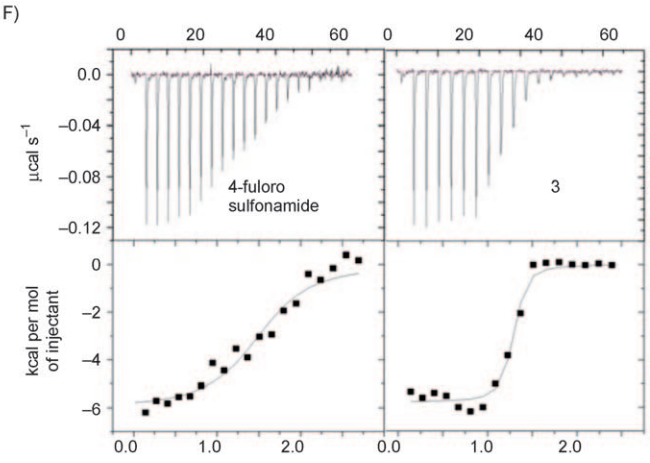
Under these considerations, we designed and synthesized compound **3**, which is derived from the structural features of both **C** and **F10** (Figure 3D). We used isothermal titration calorimetry (ITC) to characterize the binding affinities of this synthesized compound and derivatives **4**–**6**, which were also synthesized to explore the contributions of the pyridine and piperazine moieties to the binding affinity (Figure 3E). Fragment **C**, 4-fluorobenzene sulfonamide, was shown to bind to the protein with a K_d of 435 nM (Figure 3E and 3F). Compounds **4** and **5**, which have a piperazine moiety but do not have a pyridine moiety on the structure, showed similar affinities (K_d = 426 nM and 208 nM, respectively). Compound **6**, which has a ketone linkage from the piperazine ring to the pyridine ring, showed a relatively strong affinity (K_d = 100 nM). Importantly, designed compound **3** showed the strongest binding affinity to CA (K_d = 29 nM; Figure 3F and E). These binding affinity strengths are consistent with the IC_{50} values that were obtained by an enzyme inhibitory assay for CAII (Figure 3E). Furthermore, **3**, which was identified by FCA was employed for docking analysis with CAII by using the Builder module of the Insight II program. As expected, the docking configuration of the inhibitor–enzyme complex revealed that **3** fits well along the binding groove of the enzyme's active site (Figure 3G). In this model, Phe131 formed π–π stacking interactions with a pyridine ring of **3**. The effect was expected to contribute to the overall binding potency of **3** to the protein.

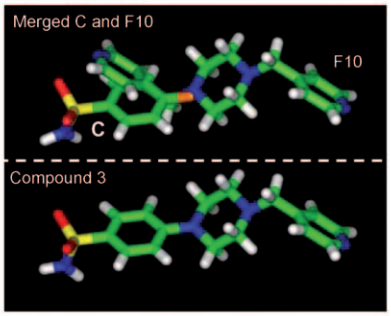
Fragment-based ligand discovery has become one of several new significant approaches for conventional drug discovery. Although a large variety of methods have been used,[1–3] screening of useful fragments for final candidate discovery is often hampered by the throughput of the available systems. Surface plasmon resonance (SPR)-based ligand screening has been reported to be a powerful tool for studying biomolecular interactions, such as those between compounds and proteins on a metal surface (for example, gold film).[14a–c] This approach has also proven to be suitable for fragment-based ligand discovery by identifying several fragments that bind to target protein weakly by using fragment libraries of up to 100000 compounds in a high-throughput manner by scientists at Graffinity Pharmaceuticals.[3b,14d–f] The SPR-based fragment screening requires the preparation of a library of compounds that are tagged with several linker moieties that contain the so-called "ChemTag," to come into contact with a gold film. Their signal detection system on chip does not require the proteins to be labeled, but it does require them to be purified, as is the case with other techniques. In contrast, when FCA is used in the screening, a pair of fragments is only mixed in various combinations during the preparation of the fragment library. Screening by FCA tests a pair of fragments for the target protein. Hence, two fragments that are detected by FCA could be accessed on the proximal pockets of the protein; therefore, combining the two fragments should be a faster route to higher-affinity ligands. Additionally, the use of cell lysates is motivated by the fact that mammalian proteins that are expressed in mammalian cells properly retain the protein's functions and are prepared easily without tedious purification steps of the protein. On the other hand, the FCA approach has remaining issues to be considered. For example, in Figure 3B, affinities of the four fragments, **A**–**C** and **E** for CAII should be enough to be detected alone and as mixed spots with significant fluorescence signals (Table S1). We have identified more low-affinity binders with micromolar-range affinities for target proteins on a photoaffinity slide by using cell lysates. Hence, we think that the lack of significant signals of these four fragments is not due to a problem of affinity limit but of a hindrance by linker moieties. When fragments with a low molecular weight are conjugated to the phenyldiazirine group of photoaffinity linker through photogenerated carbene species, it is difficult for the target protein to recognize these small molecules by separating them from chemical structures of phenyldiazirine groups of the linker parts. This is supported by the fact that acetazolamide, a well-known CAII binder with a low molecular weight and a high affinity (K_d = 31 nM, M_W = 222.25),[15] did not show a significant signal on the chip (data not shown). Difficulties in detecting molecules with a low molecular weight may remain as one of the key issues to be clarified with chemical array screening techniques.[16] The concept of a mixed fragment

Figure 3. A) Structures of selected anchor fragments **A**–**E**, and block fragments **F1**–**F10**. B) Fluorescent slide image by scan after treatment with cell lysates that expressed RFP-fused CAII. In a demonstration of FCA, five anchor fragments (**A**–**E**) were immobilized in the presence of various block fragments (**F1**–**F10**); white bar, 800 μm. C) The fluorescence signal of the FCA is shown. The total intensity was corrected for background intensity. The error bars denote the standard deviation of three replicates. D) Compound **3** was designed by a merge on a computer by using a pair of binding fragments, **C** and **F10**. E) Inhibitory activity of the compounds for CAII was measured.[13] The IC_{50} values were calculated from independent triplicate experiments. K_d values were obtained by ITC analysis. F) ITC profiling of fragment **C** and synthesized derivative **3** for CA. A solution of ligands (10 μM) in Tris–SO_4 buffer containing 4% (*v*/*v*) DMSO was titrated with a solution of the protein (130 μM) in the same buffer. G) The model of CAII–**3** by using the Biopolymer and Discover3 modules of Insight II; nitrogen: blue, oxygen: red, sulfur: yellow. Mixed solutions of these compounds were prepared with every combination between anchor and block fragments. Thus, there were 50 combinations of anchor fragments **A**–**E** and block fragments **F1**–**F10** on the array slide. The slide was incubated with cell lysates that overexpressed RFP-fused CAII, and fluorescence signals were detected on the array slide by a microarray scanner (Figure 3B). The points of intersection of each fragment on the lengthwise (**A**–**E**) and crosswise lines (**F1**–**F10**) showed that combinations of the two ligands were immobilized in a mixed manner on the slide. The outside lines are areas spotted by these fragments alone as a control for combination effects. On the array figure, a binding signal was clearly observed on a mixed spot of fragments **C** and **F10** (Figure 3B) with highest fluorescence signals compared with signals of other pairs (red bar; Figure 3C).

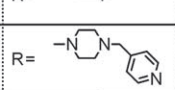
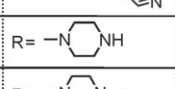
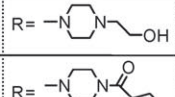
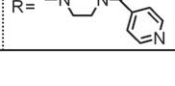


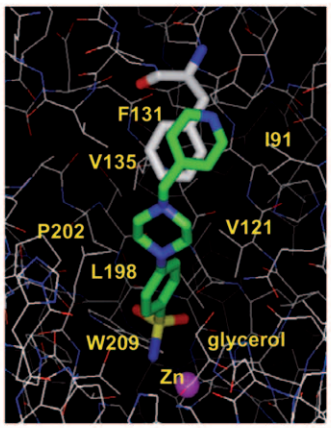






E)

Compounds	H_2NO_2S–R	K_d / nM	IC_{50} / nM
4-fuloro sulfonamide	R= –F	435	203
3	R= 	29	48
4	R= 	426	299
5	R= 	208	137
6	R= 	100	72



should help to remove these limitations, and make possible the detection of fragment pairs that could not be identified alone over the traditional chemical array screen. The FCA approach is better suited for larger libraries of fragments rather than the small library that was used in the CAII inhibitor screen. Further work is underway to clarify the molecular weight limitations and to identify high-affinity bivalent ligands of proteins of interest by increasing the number of combinations of fragments in our laboratory.

In conclusion, we describe a novel approach in which the concepts of fragment-based ligand discovery are combined with chemical array techniques to yield bivalent inhibitors. The utility of our method was demonstrated by the detection of interactions of known FKBP12 binders and FKBP12, and yielded a novel bivalent ligand for CAII with high affinity. Importantly, the method does not require large quantities of purified protein or labeling of small molecules to identify the interactions. Hence, the method is relatively simple and paves a new way in traditional fragment-based approaches by capitalizing on the powerful capability that is offered by the chemical array platform.

Experimental Section

Chemical syntheses of **1**–**6** are reported in the Supporting Information.

Preparation of fragment combination array slides: A solution of fragments was mixed on a plate (final concentration of each compound, 10 mM in DMSO). The photoaffinity-linker-coated glass slides were prepared according to our previous reports by using DNA microarray TYPE1 slides (high-density mine-coated slides; cat. No. S159515 Matsunami Glass, Tokyo, Japan).[6b,7b] A solution of the compounds in the plate was spotted onto the glass slides with an automated spotter (TOYOBO, Tokyo, Japan). After that, the slides were dried in vacuo to evaporate the DMSO. The slides were exposed to UV irradiation of 4 J cm^{-2} at 365 nm by using a CL-1000 L ultraviolet crosslinker (UVP, Upland, CA, USA). They were then washed successively with DMSO, DMF, THF, EtOH, and Milli-Q H_2O and dried.

Treatment of glass slides with cell lysates and scanning of slides for fluorescence: Preparations of mammalian cell lysates that express RFP-fused proteins were performed with HEK293T cells.[7b] Overexpression of the RFP-fused protein was confirmed by using a fluorescence scanner or Western blotting. Glass slides were incubated for 1 h at 4 °C with cell lysates overexpressing RFP-fused FKBP12 or CAII. After incubation, the slides were washed briefly, dried, and scanned at 532 nm on a GenePix microarray scanner (Amersham Biosciences). The fluorescence signals were quantified with GenePix 5.0 software.

Isothermal titration calorimetry and CAII inhibition: Isothermal titration calorimetry (ITC) experiments were done at 25 °C by using a MicroCal iTC200 (MicroCal, Northampton, MA, USA). A ligand solution (10 μM) in Tris–SO_4 buffer (pH 8.4) that contained 4% (*v*/*v*) DMSO was titrated with 130 μM of CA (Sigma) in the same buffer. After an initial dummy injection (0.2 μL), the injection profile of ligand solution (2 μL) into the calorimeter cell was performed. The ITC profile of fragments **F1**–**F10** and **D** was not detected by ITC analysis under the ligand-soluble conditions, because the binding constant was near the upper limit of direct titrations. The resulting titration curves were then processed and fitted with Origin 7 software. Inhibition activity of the compounds for CAII was measured with 4-nitrophenylacetate as the substrate, which allowed for detection of the esterase activity of the enzyme. Ligands and the CAII solution (final 50 nM) were incubated for 10 min in 50 mM Tris–SO_4 buffer (pH 8.4). After the addition of a solution containing of 4-nitrophenylacetate (final 10 mM), the reaction was started and monitored with a Wallac ARVOSX plate reader (PerkinElmer) at 405 nm. Nonenzymatic hydrolysis rates were subtracted from the observed rates, and the IC_{50} values were calculated from independent triplicate experiments.

Modeling: Structural construction of fragments **C** and **F10** and compound **3** were carried out by the Builder module of the Insight II program (Accelrys Inc., San Diego, CA). The atomic coordinates of human CAII were taken from the X-ray structure of the CAII–*p*-aminoethylbenzenesulfonamide (pAEBS) complex (PDB ID: 2NNG[17]). The model of the CAII–**3** complex was constructed by using the Biopolymer and Discover3 modules of Insight II. The benzenesulfonamide moiety of **3** was graphically superimposed on the same moiety of pAEBS. In energy minimization and molecular dynamics calculations, the CVFF force field was used. The initial CAII–**3** structure was subjected to 2000 rounds of energy minimization to remove bad contacts within the complex. After a 3 ps molecular dynamics at 300 K, the resulting structure was further energy-minimized in 2000 steps.

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