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Photo-Cross-Linked Small-Molecule Microarrays as Chemical Genomic Tools for Dissecting Protein–Ligand Interactions

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Abstract: We have developed a unique photo-cross-linking approach for immobilizing a variety of small molecules in a functional-group-independent manner. Our approach depends on the reactivity of the carbene species generated from trifluoromethylaryldiazirine upon UV irradiation. It was demonstrated in model experiments that the photogenerated carbenes were able to react with every small molecule tested, and they produced multiple conjugates in most cases. It was also found in on-array immobilization experiments that various small molecules were immobilized, and the immobilized small molecules retained their ability to interact with their binding proteins. With this approach, photo-cross-linked microarrays of about 2000 natural products and drugs were constructed. This photo-cross-linked microarray format was found to be useful not merely for ligand screening but also to study the structure–activity relationship, that is, the relationship between the structural motif (or pharmacophore) found in small molecules and its binding affinity toward a protein, by taking advantage of the nonselective nature of the photo-cross-linking process.

Keywords: immobilization · ligand screening · microarrays · small molecules · structure–activity relationships

Introduction

The identification of small-molecule modulators for a protein of interest can facilitate not only the functional analysis of the protein but also the development of clinical drugs when the protein is associated with human diseases. A large number of both naturally occurring and synthetic small molecules that modulate specific cellular proteins and thereby specific biological processes have been discovered.[1,2] In particular, natural products have been an important resource for such compounds. For example, the polyketide compound reveromycin A[3,4] inhibits protein synthesis in mammalian cells and induces apoptosis specifically in osteoclasts by blocking the aminoacylation activity of isoleucyl-tRNA synthetase,[5] and the immunosuppressant drugs rapamycin[6] and FK506,[7] which strongly bind to FKBP proteins,[8,9] were originally identified as secondary metabolites of the genus *Streptmyces*. Similarly, amphidinolide H,[10] a macrolide that binds to actin in a 1:1 stoichiometry and thereby disrupts the actin organization in mammalian

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cells,[11] was discovered by screening dinoflagellate products for compounds with antiproliferative properties against cancer cells.

A considerable number of these bioactive natural products are known to modulate their macromolecule targets through specific molecular recognition[12] and direct binding.[13] Recently, a growing number of research laboratories are doing biological research with such small-molecule probes, so-called bioprobes,[1] which are capable of yielding insight into the role of individual macromolecules in complex biological systems. High-throughput assay formats were recently created to screen such small-molecule ligands and modulators for proteins of interest.[14]

Small-molecule microarrays represent one of the most promising approaches developed to date.[15,16] In general, solid surfaces are derivatized with certain functional groups (functional group B in Figure 1a). Compounds with a reactive functional group (A) readily attach to the surface upon printing through a selective-coupling reaction. The resulting arrays of small molecules are used to screen ligand molecules for individual proteins. Recently, Schreiber and co-workers reported several successful results in which the ligands identified were used to dissect the biological functions of the protein.[17,18]

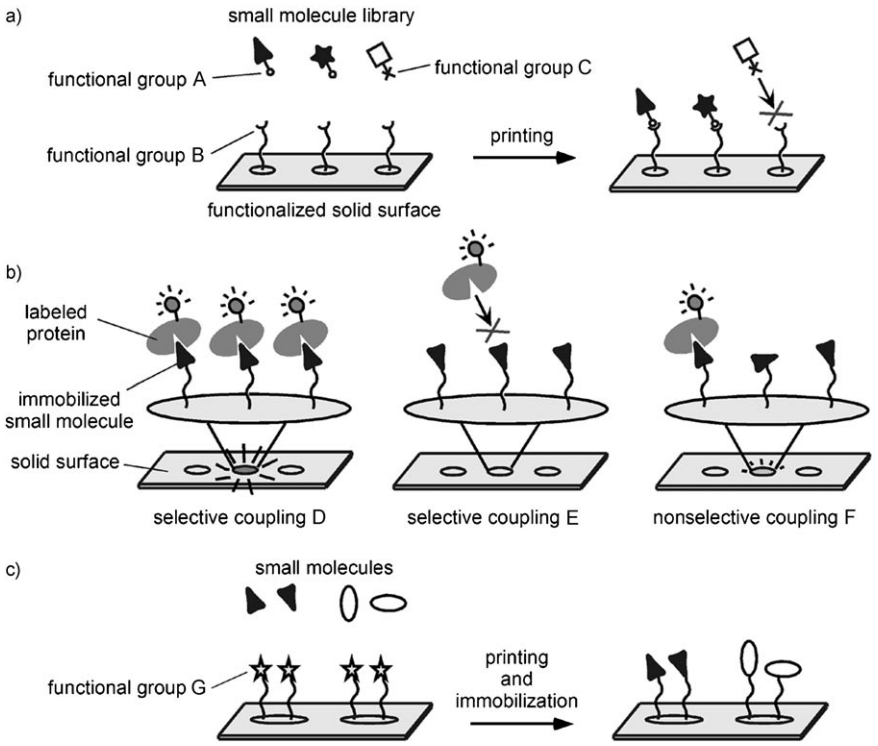

Figure 1. Overview of small-molecule microarrays. a) Upon printing, a library of small molecules with functional group A readily attach to the solid surface that is derivatized with functional group B, which is reactive to A. Coupling between an inadequate pair of functional groups (i.e., B and C) does not occur. b) When the small molecules are properly immobilized through a selective coupling (D), interactions between these molecules and their binding proteins can be detected sensitively. However, when the immobilization is inadequate (e.g., E), the immobilized small molecule is useless for the protein binding assay. Nonselective immobilization (F) enables not only the introduction of a variety of small molecules, but also the production of a variety of conjugates, some of which are expected to retain affinity toward the binding protein. c) The ideal functional group (G) should be activated under mild conditions, and the resulting species should immobilize a variety of small molecules in a functional-group-independent manner. Moreover, the functional group should not remain on the surface after the immobilization process.

Although the selective-coupling approach is very suitable for immobilizing synthetic compounds, natural products are structurally diverse and thought to be difficult to introduce onto a slide surface in a uniform manner. Naturally occurring small molecules, which include terpenes, polyketides, peptides, alkaloids, and their conjugates, do not have a common "handle" for immobilization that can be easily designed and introduced onto each synthetic ligand.[19] Therefore, a conceptually different approach should be developed to introduce natural products onto a solid surface. Indeed, several groups have recently reported their own approaches.[20,21]

There remains another hidden drawback to using the selective-immobilization approach (Figure 1b). Even though small molecules, whether naturally occurring or synthetic, can be successfully introduced onto solid surfaces by using a selective coupling reaction, part of the small molecule faces the solid surfaces, and therefore some of the immobilized small molecules will not come into contact with the relevant binding proteins. In other words, as MacBeath et al. noted

Abstract in Japanese:

トリフルオロメチルアリールジアジリン（TAD）基の光分解により生成するカルベン種を利用した、新規低分子（小分子）マイクロアレイの作成法を開発した。まず、我々は、TAD 基の光分解で生じるカルベン種が様々な低分子有機化合物とクロスリンクすること、また、このカルベン種がそれぞれの化合物と多様なコンジュゲートを生成することを見出した。次に、この方法を用いてガラス基板上に固定化された化合物群が、それぞれの特異的結合タンパク質との結合能を保持していることを確認した。更に、天然有機化合物を含む、約 2000 種類の化合物群を固定化した低分子マイクロアレイを作成し、本プラットフォームがタンパク質のリガンドスクリーニングのみならず、結合タンパク質が認識する低分子化合物上の部分構造の特定に有効であることを示した。本マイクロアレイ化法は、既存の方法とは異なり、固定化される化合物上に特定の官能基を必要としないため、低分子マイクロアレイのプラットフォームに天然物を含む様々な低分子化合物を利用可能とした点が画期的であり、低分子マイクロアレイ技術の一般化に大きく道を開くものである。

in their first report on small-molecule microarrays,[15] the presence of a linker connecting the small molecule to the solid surface decreases the number of binding modes available to each compound. Although the resulting arrays enable the selection of small molecules with known sites of attachment, which can be useful tools for affinity purification and for binding kinetics, the decrease in the number of binding modes poses a major drawback to ligand screening and chemical genomics,[22] especially when the goal is to discover a new type of ligand for a protein with unknown function and structure.

To overcome these drawbacks for the selective-immobilization approach, we started to develop a conceptually new "nonselective" immobilization that enables the introduction of a variety of small molecules to solid surfaces, and found that photogenerated carbene species can cross-link a variety of small molecules on a solid surface in a functional-group-independent manner.[23] Herein, we give a full account of this investigation, which led to the construction of photo-cross-linked natural-product microarrays. We also describe herein the possibility of the platform as a tool for on-array analyses of the structure–activity relationship (SAR) of ligand-binding specificity toward a binding protein.

Results and Discussion

Trifluoromethylaryldiazirine as a Useful Functional Group for Photo-Cross-Linking of Small Molecules

We considered that an ideal functional group on a solid support to immobilize structurally diverse small molecules in a functional-group-independent manner should satisfy the following criteria (Figure 1c). 1) It should be possible to activate the functional group under mild conditions. In other words, none of the small molecules to be immobilized should degrade under the conditions that are used to activate the reactive group, if possible. 2) Preferably, the reactive species generated should react with all possible functional groups on the small molecule to afford a large number of conjugates, even though the small molecule is composed of hydrogen and carbon. 3) The reactive species should not remain on the solid surface after the immobilization process. If it remains, the species can react with proteins during a binding experiment, resulting in nonspecific protein binding and thereby a high background level in the binding assay.

To address these requirements, we focused on photoactivatable functional groups as reagents to be introduced onto solid surfaces. Photoreactive groups such as aryl diazirines, aryl azides, and benzophenone derivatives are known to become highly reactive species such as carbenes, nitrenes, and biradicals, respectively, upon UV irradiation.[24,25] These species are so reactive that they can react with (or insert into) a heteroatom–hydrogen or even a carbon–hydrogen bond. These types of photoreactive groups are usually introduced onto ligand molecules through an appropriate linker and are used in photoaffinity labeling experiments for analyzing binding sites within receptor molecules. Although compounds such as polyenes are damaged by irradiation with UV light, UV-C irradiation can be thought of as one of the mildest conditions available.

Among the photoreactive groups, we selected trifluoromethylaryldiazirines (TADs)[26] as the reagents of choice. The photogenerated carbenes from TADs are highly reactive toward a variety of chemical bonds, including $C(sp^3)$–H, $C(sp^2)$–H, O–H, C–Cl, N–H, Si–H, and C=C double bonds.[27–29] Furthermore, they have a low tendency for ring expansion, whereas the related aryl nitrenes tend to do so over an energy barrier of several kcal mol^{-1} to form a species called ketenimine, which is only reactive toward nucleophiles.[30] Also, a linear diazo isomer, a side product of the photolysis of TADs, was shown to be reasonably stable owing to the presence of a trifluoromethyl group,[26] whereas diazo compounds are highly sensitive to protonation and subsequent nucleophilic attack in general.[31,32] The stability of the diazo isomer should decrease the side reactions that do not involve carbene intermediates and the nonspecific protein binding.

To test whether TADs can be used to immobilize (or cross-link) a variety of small molecules on a solid surface, photo-cross-linking experiments between small molecules and TADs were examined. A linker molecule **1**, which we named the photoaffinity linker, was designed and prepared from *N-tert*-butoxycarbonyl-2,2′-ethylenedioxybis(ethylamine) and 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoic acid (Figure 2).[23] We expected that the terminal amine of **1** can be attached to the slide surfaces in various ways, and the ethylene glycol moiety was expected to decrease nonspecific absorption of proteins onto **1** as well as solid surfaces functionalized with **1**.

Ten types of structurally diverse and commercially available small molecules (rhodamine B, nocodazole, colchicine, benomyl, digoxin, digitoxin, hydrocortisone, beta-estradiol, progesterone, and cyclosporin A) were independently mixed with **1** (0.1 equiv for the respective small molecules) in MeOH.[33] The solutions were concentrated and dried in vacuo, a step that was expected not merely to maximize the efficiency of photo-cross-linking between small molecules and **1**, but also to avoid the cross-linking of the organic solvent with **1**. After the resulting semisolid residues were irradiated with a UV-C lamp, the mixtures were redissolved in MeOH and analyzed by an LC ESI MS spectrometer.

The results of photo-cross-linking between small molecule digoxin (**2**) and **1** are shown in Figure 2. Upon irradiation, multiple peaks of the molecular weight of the cross-linked products were observed besides the peaks derived from the linear diazo isomer **3** and some side products (Figure 2b). Notably, as shown in Figure 2b, we observed conjugates in every experiment and multiple peaks derived from the cross-linked conjugates in most cases. Although it was difficult to determine which peak had what structure, and this model experiment may not reflect events on the solid surface, the results gave us an intuitive expectation that the TAD group of **1** can be used to cross-link a variety of small

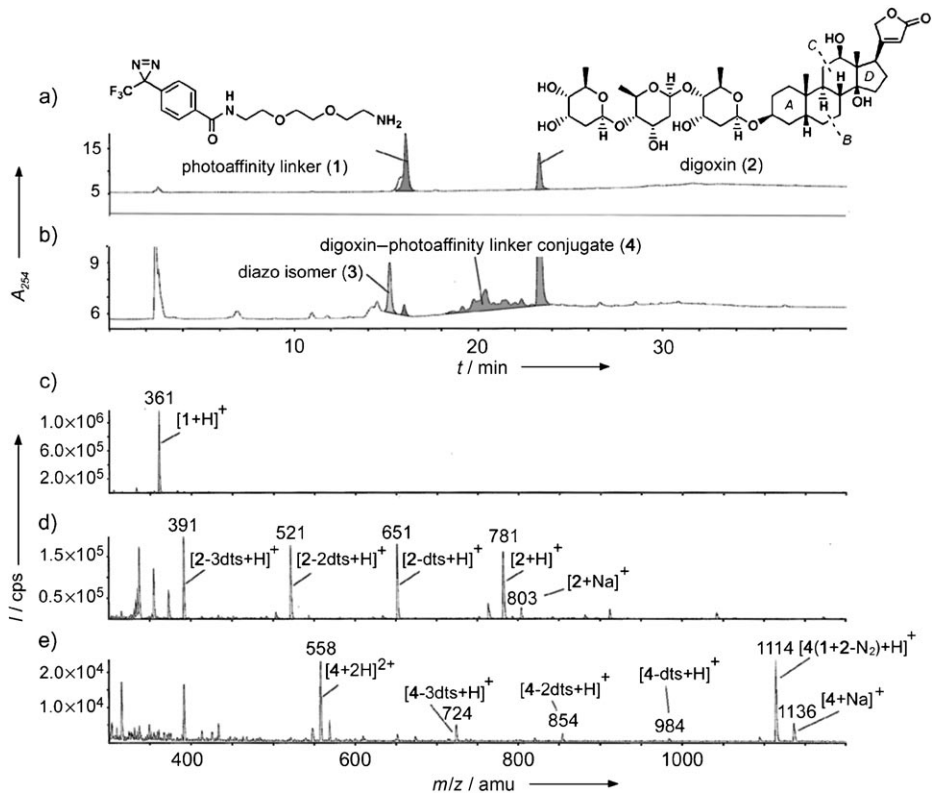

Figure 2. Detection of photo-cross-linked conjugates between photoaffinity linker and digoxin by liquid chromatography (LC) ESI MS analysis. a) LC data for a mixture of photoaffinity linker and digoxin; b) after photo-cross-linking (365 nm); c–e) ESI MS data for photoaffinity linker (c), digoxin (d), and digoxin–photoaffinity linker conjugate (e). LC data were recorded at 254 nm. A_{254} = absorbance at 254 nm, dts = digitose.

molecules in a comparatively random orientation, which cannot be accomplished with the selective approach. Furthermore, we recently performed the photo-cross-linking experiments of **1** in several lower alcohols at the freezing temperature, and found that the photogenerated carbenes from **1** can insert into every possible C–H and O–H bond in a relatively uniform distribution in the solid state.[34]

Preparation of Photo-Cross-Linked Small-Molecule Macroarrays: Proof-of-Concept Studies[23]

Highly nonselective reactions are usually recognized to be totally useless and unsophisticated in the community of organic chemists. There is no doubt that selectivity in all respects (chemo-, regio-, stereo-, and enantioselectivity) has been, and will be, a key issue in modern organic chemistry.[35,36] However, as we described above, the nonselective reaction has an advantage in terms of universality and product diversity. Thus, it is possible that the question is not the nonselectivity of the reaction itself, but whether the "signal" (e.g., a binding signal between a conjugate and a protein in a binding assay) can be extracted and detected from the big mixture that results from the nonselective reaction.

To answer the question, we designed photoaffinity-linker-coated (PALC) glass slides, which can be prepared by introducing **1** onto amine-coated glass slides through a urea linkage. We expected that small molecules can be immobilized on the PALC glass slides by photo-cross-linking, and that the immobilized small molecules (more correctly, some conjugates from the photo-cross-linked small molecules) retain binding affinity toward possible binding proteins, thus giving detectable fluorescence signals when the proteins are fluorescently labeled.

Seven natural products (biotin, rapamycin, digoxin, digitoxin, cyclosporin A, digoxigenin, and FK506), which are known to bind known and specific proteins,[15] and fluorescent rhodamine B were dissolved in dimethyl sulfoxide (DMSO) at various concentrations (0.01–100 mM), and the solutions were spotted in quadruplicate (0.2 μL each) on the PALC macroarray glass slides (spot diameter 1 mm). These spots of small molecules were dried in vacuo and photo-cross-linked on the PALC glass slides. It is expected that the high concentration of, and thereby the excess number of, small molecules on the surface should encircle each TAD group on the slide and decrease the cross-linking within **1**, the intermolecular cross-linking between two molecules of **1**, and the multiple cross-linking of **1** with a small molecule. The resulting photo-cross-linked small-molecule macroarrays were washed thoroughly with organic solvents and used for protein binding assays.

As a consequence of direct observation of macroarrays with a fluorescence scanner, it was shown that the immobilization of rhodamine B depends on the following factors: 1) the presence of the photoaffinity linker **1**, 2) the concentration of the small-molecule solution, 3) the amount of irradiation, and 4) the transparency of the small-molecule layer that covers spotted areas of the slide. The results strongly suggest that the expected immobilization (i.e., photo-cross-linking) did occur on the solid surface. Furthermore, the photo-cross-linked compounds were successfully recognized in a specific manner by their binding proteins (Figure 3).[37] This is the first example of the immobilization of complex natural products on a solid surface as arrays in a uniform process. Moreover, the binding signals were successfully extracted from the mixture with excellent signal-to-noise (S/N) ratios.

Notably, a similar photo-cross-linking immobilization of biomacromolecules on solid supports by using aryl nitrenes

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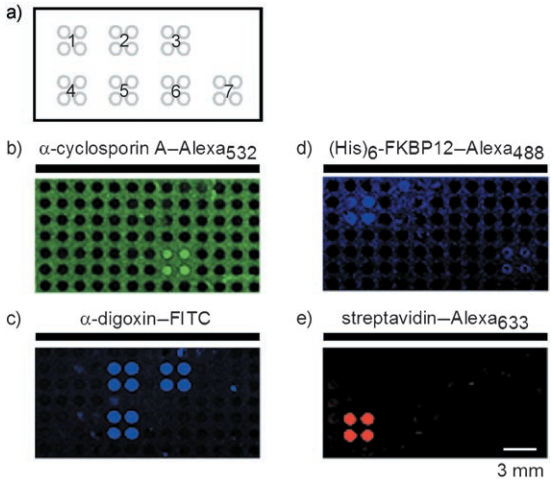


Figure 3. Detection of interactions between photo-cross-linked small molecules and labeled proteins. a) Array design: 1 = rapamycin, 2 = digitoxin, 3 = digoxigenin, 4 = biotin, 5 = digoxin, 6 = cyclosporin A, 7 = FK506. b–e) Fluorescence images of the slides: b) probed with anti-cyclosporin A antibody–$Alexa_{532}$ conjugate (10 $\mu g\,mL^{-1}$) and scanned for $Alexa_{532}$; c) probed with anti-digoxin antibody–FITC conjugate (150 $\mu g\,mL^{-1}$) and scanned for FITC (FITC = fluorescein-isothiocyanate); d) probed with $(His)_6$-FKBP12–$Alexa_{488}$ conjugate (10 $\mu g\,mL^{-1}$) and scanned for $Alexa_{488}$; e) probed with streptavidin–$Alexa_{633}$ conjugate (5 $\mu g\,mL^{-1}$) and scanned for $Alexa_{633}$.

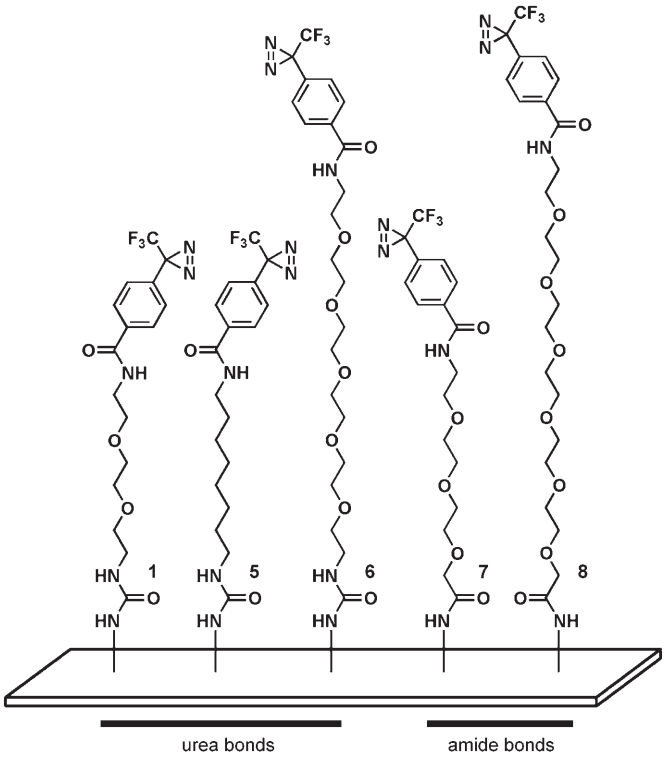


Figure 4. Comparison of photoaffinity linkers with a different length, hydrophilicity, and connection mode between linkers and slide surface.

and aryl carbenes was previously reported,[38–40] but our results are thought to be significant because they demonstrate that the methodology can be applied to the small-molecule level.

Construction of Photo-Cross-Linked Compound Microarrays

The results of the proof-of-concept studies with the macroarray platform prompted us to construct photo-cross-linked small-molecule microarrays from a large library of natural products. For this purpose, we used a Spectrum Collection (Microsource Discovery Systems Inc., USA) that consists of 2011 natural products and drugs. A map for the microarrays was designed as shown in the Supporting Information. In brief, the 2011 compounds were spotted in duplicate on a single slide, so that the binding signals and noise (tiny scratches or patches) could be easily distinguished by comparing the two arrays. Conversely, when the binding signals were observed in the same positions for the two different arrays, we could define them as "hit" signals, whether positive or false-positive.

With this microarray platform and the proteins used in the proof-of-concept studies, we reoptimized the length and hydrophilicity of the linker connecting TAD groups and the slide surface, as well as the coupling method used to introduce the amine linker onto the slide surface (Figure 4). Among the photoaffinity linkers (**1**, **5**–**8**) and coupling methods evaluated, the combination of linker **6** and a urea linkage gave optimal results (data not shown). Figure 5 shows the fluorescence image of the photo-cross-linked small-molecule microarray, which was blocked with 1% skimmed milk and probed successively with $(His)_6$-FKBP12 (2 $\mu g\,mL^{-1}$), polyclonal rabbit anti-$(His)_6$ antibody (MBL Co. Ltd., Nagoya, Japan) (10 $\mu g\,mL^{-1}$), and $Alexa_{633}$-labeled anti-rabbit IgG antibody (Invitrogen Co., CA, USA) (100 $\mu g\,mL^{-1}$). Binding signals were clearly observed on the FK506- and rapamycin-spotted areas, and a good S/N ratio was obtained.[41]

Interestingly, although the introduction of photoaffinity linkers **7** and **8** through an amide linkage improved the density of photoaffinity linkers to 3–5 $pmol\,mm^{-2}$,[15,42] which is at least 30-fold higher than the density of **6** that was introduced onto the slide through a urea linkage, neither the intensity nor the S/N ratios of the observed binding signals were improved.

Besides the methods for probing slides described above, several protocols can be applied to observe small-molecule–protein interactions. In particular, the tyramide signal amplification (TSA) system (PerkinElmar, MA, USA), an enzyme-mediated detection method that utilizes the catalytic activity of horseradish peroxidase (HRP) to generate high-density labeling of a target protein,[43] can be applied to the small-molecule microarray platform, usually resulting in superior S/N ratios.

In an attempt that involved a library of various peptides and the resulting photo-cross-linked peptide microarrays, we noticed that the immobilized phosphotyrosyl peptide–biotin conjugates (biotin–ABBBVHpYARLI; B = β-Ala) recog-

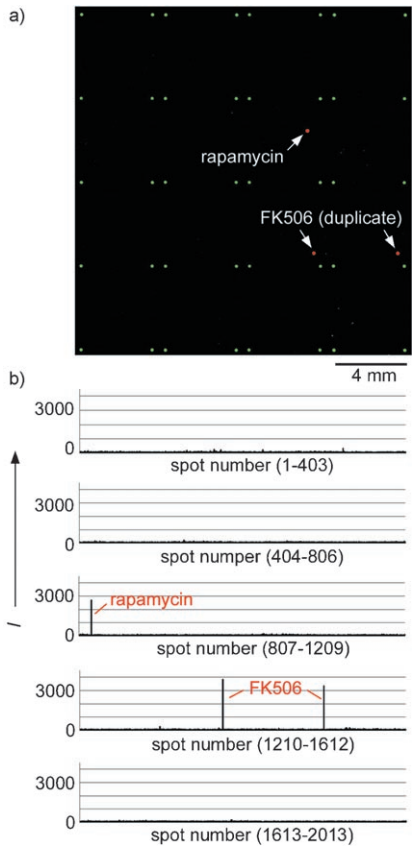


Figure 5. On-array detection of interactions between FKBP12 and FKBP binders. a) Fluorescence image of the slide probed successively with $(His)_6$-FKBP12 (2 μg mL^{-1}), rabbit anti-$(His)_6$ antibody (10 μg mL^{-1}), and mouse anti-rabbit IgG antibody–$Alexa_{633}$ conjugate (100 μg mL^{-1}). Two spots of FK506 were added and printed as positive controls. Green signals = position markers (rhodamine B). b) Plot of fluorescence intensity for each spot.

nized not only streptavidin but also the SH2 domain of protein tyrosine phosphatase SHP-1, with dissociation constants (K_D) in the femtomolar and low micromolar range, respectively (see Supporting Information).[44] This result suggests that the immobilization took place both on biotin and on the peptide moiety, thus demonstrating again the functional-group independency of the photo-cross-linking process.

SAR on the Photo-Cross-Linked Small-Molecule Microarrays

When the photo-cross-linking protocol is used, it is expected, as described above, that the immobilized small molecules will display virtually all of their surfaces, thus giving them the inherent ability to interact with their binding proteins. This also means that it is possible to study the SAR, that is, the relationship between the structural motif (or pharmacophores) found in small molecules and the binding affinity of the motif toward a protein of interest.

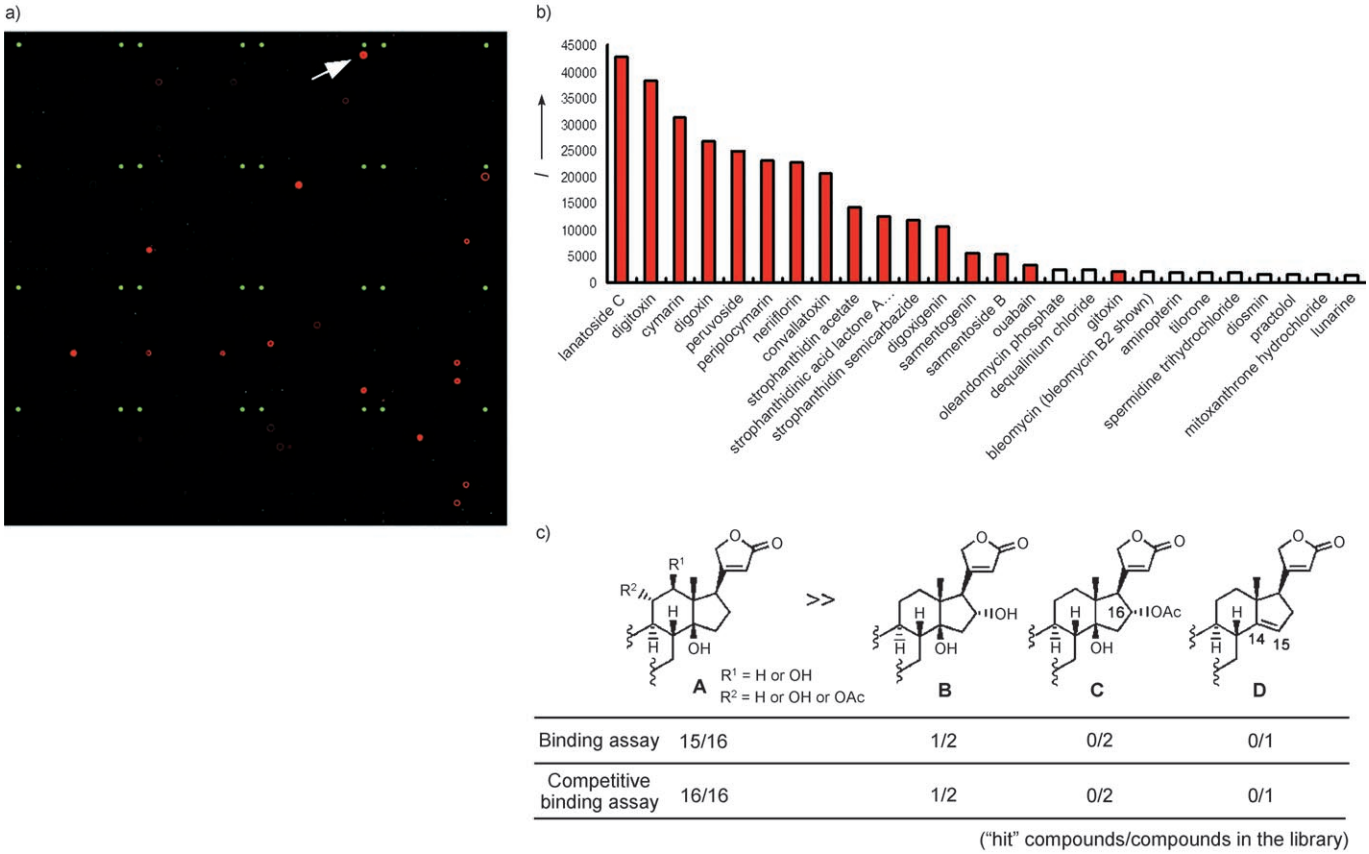
When the photo-cross-linked small-molecule microarrays were treated with a monoclonal mouse anti-digoxin antibody (2 μg mL^{-1}; Sigma), and binding was detected with an $Alexa_{633}$-conjugated anti-mouse IgG antibody (100 μg mL^{-1}), many binding signals, including signals on digoxin, were observed (Figure 6a). Most of the "hit" compounds on whose spots strong signals were observed were steroids and steroidal glycosides, each of which had a butenolide on the D ring (for the structure of digoxin, see Figure 2; see also Supporting Information for all structures of "hit" compounds). In fact, we also observed fluorescence signals on other molecules (Figure 6b), but in contrast to the signals on the steroids, these signals were observed when the slide was treated directly with $Alexa_{633}$-conjugated anti-mouse IgG antibody (see Supporting Information). These signals did not increase when the concentration of the anti-digoxin antibody was increased. This result showed that these false-positive signals originated from the fluorescence of the compounds themselves and/or from binding with Alexa-labeled antibody.

The C-, D-, and butenolide-ring regions of the "hit" compounds are closely related to each other, thus indicating the possibility that the antibody recognizes the structure. Interestingly, other structurally related steroidal compounds were found in the 2011 library, although only very weak binding signals were observed for them (see Supporting Information for all compounds containing butenolide). Five out of 21 butenolide-containing steroidal compounds did not give significant binding signals (Figure 6c). Analysis of these structures revealed that the presence of the C16 α-acetoxy group (group C in Figure 6c) or a C14–C15 double bond (group D) had a totally negative effect on binding. The C16 α-hydroxy group (group B) also tended to have a negative effect. Finally, the compound strophanthidin (see Supporting Information), which does not contain these structural features, did not give a strong signal.

We then carried out the competitive binding assay on the microarrays (see Supporting Information). This experiment gave us information about whether the compounds on the arrays bind to the digoxin-binding region of the antibody. The anti-digoxin antibody was incubated first in the presence of various concentrations of digoxin, then the mixture was applied to the microarrays. After washing, the observed signals on the steroidal compounds were extracted and analyzed.

The results showed that fluorescence signals on the group A compounds, including strophanthidin, decreased as the concentration of the competitor increased (Table 1; see also Supporting Information). In contrast, four other compounds (groups C and D and one in group B) did not compete with digoxin. We concluded from the array experiments that the antibody recognizes mostly a domain that consists of C, D, and butenolide rings, and that the C14–C16 portion of the molecules is critical to the binding. This working hypothesis was confirmed by a solution-phase competitive binding assay with [^{3}H]digoxin (Table 1; see also Supporting Information). The experimental results in solution were in good agreement with the array results.

Very recently, Schreiber, Koehler, and co-workers reported the expanded functional-group compatibility of their



	A	B	C	D
Binding assay	15/16	1/2	0/2	0/1
Competitive binding assay	16/16	1/2	0/2	0/1

("hit" compounds/compounds in the library)

Figure 6. SAR on the photo-cross-linked small-molecule microarrays. a) A fluorescence image of the slide probed with mouse anti-digoxin antibody (2 $\mu g\,mL^{-1}$) followed by anti-mouse IgG antibody–$Alexa_{633}$ conjugate (100 $\mu g\,mL^{-1}$). The white arrow indicates the spot on which digoxin was immobilized. b) Plot of fluorescence intensity of the immobilized small molecules, listed in order of magnitude. Red rectangles indicate the fluorescence signals that varied depending on the concentrations of both anti-digoxin antibody and competitor (digoxin). White rectangles indicate those signals that did not vary with these concentrations. c) Structural trend of steroidal compounds for anti-digoxin binding. The A and B rings of each steroidal structure are omitted for clarity. The "hit" compounds are the compounds for which significant fluorescence intensity (> (mean of background signals + 9 × standard deviation of background signals)) is observed.

Table 1. Relationship between on-array and solution-phase binding assays. The 11 selected compounds are listed in order of fluorescence signal intensity. The symbols "+" and "±" indicate the degree of competition in the competitive binding assay on arrays. Details of the results are summarized in the Supporting Information.

Compound	Category	Binding[a]	Competition[a]	IC_{50} [nM][b]
lanatoside C	A	42825	+	5
digitoxin	A	38176	+	19[c]
digoxin	A	26647	+	3[c]
digoxigenin	A	10454	+	6[c]
sarmentogenin	A	5644	+	205
ouabain	A	3206	+	1628[c]
gitoxin	B	2099	+	204
oleandrin	C	779	±	4300
gitoxigenin	B	736	±	> 20000
gitoxigenin diacetate	C	725	±	> 20000
anhydro-β-sarmentogenin	D	715	±	5750

[a] On-array binding assays. [b] Solution-phase binding assays. [c] The compounds were purchased from other vendors and used for the radioimmunoassay. See Experimental Section for details.

small-molecule microarrays to include immobilization of a variety of reactive functional groups on the surface of a single slide.[45] However, our microarray platform still has the merit that no such reactive functional group is needed for small molecules to be immobilized. For example, we successfully observed a binding event between progesterone and its antibody on the photo-cross-linked small-molecule microarrays (see Supporting Information for the detailed analysis and the SAR study).

Conclusions

We have demonstrated that the photo-cross-linking approach is useful for immobilizing a variety of small molecules on a solid surface, and the resulting photo-cross-linked small-molecule microarrays can be useful tools for screening small-molecule ligands for a protein of interest. Moreover, by using the advantage of the functional-group independence of the photo-cross-linking process, it is possible to obtain information about structural motif, which is vital for binding to a protein of interest. This information is exceptionally important in the design and synthesis of multifunctional ligands that bind to several different proteins,[46] or ligands that modulate protein–protein interactions.[47,48] Al-

though X-ray crystallographic and NMR spectroscopic analyses of ligand–protein complexes also give the same type of information, the SAR on our microarray platform is unique in that it offers structural insight into the molecular-recognition event that occurs in solution with the minimum amount of both protein and ligand samples.

Further applications of this strategy toward ligand screening and the identification of binding motifs for proteins of biological importance, as well as accompanying technological developments,[49,50] are now in progress in our laboratory.

Experimental Section

Photo-Cross-Linking Experiments between Small Molecules and 1

Typically, each small molecule (10 μmol) was mixed with **1** (1 μmol) and dissolved in a 10-mL sample vial containing MeOH (1 mL). The mixture was concentrated in vacuo. The vials containing the mixtures were irradiated at 365 nm for 1 h with a Super-light model LS-D3 lamp (Irie Seisakusyo Co., Ltd., Tokyo, Japan). The mixtures were redissolved in MeOH and analyzed with a Perkin–Elmer SCIEX API 2000 pneumatically assisted electrospray triple-quadrupole mass spectrometer equipped with a Hewlett–Packard Series 1100 HPLC system (column: 200 mm × 2 mm PEGASIL ODS column (Senshu Scientific Co., Ltd. Tokyo, Japan); mobile phase: 20–100% aq. CH_3CN containing 0.05% HCO_2H; flow rate: 0.2 mL min^{-1}).

Preparation of Photo-Cross-Linked Small-Molecule Macroarrays and Protein Binding Assays

Detailed descriptions of the synthesis of **1** and the preparation of photo-cross-linked small-molecule macroarrays are given in our previous report.[32]

Slide Preparation for Photo-Cross-Linked Small-Molecule Microarray

The slides were prepared according to our previous report,[23] with the following three modifications: 1) DNA microarray TYPE 1 slides (high-density amine-coated slides; cat. No. SD00011, Matsunami Glass Industries Ltd.) were used instead of fluororesin-imprinted DNA microarray TYPE 1 slides (cat. No. S117806); 2) photoaffinity linker **6** was used instead of **1**; 3) a solution of *N*,*N*′-disuccinimidyl carbonate (100 mM) and *N*,*N*′-diisopropylethylamine (100 mM) in *N*,*N*-dimethylformamide (DMF) was used to activate the slide surfaces. The photoactivatable slides prepared were degassed and stored at −20 °C until use.

Small-Molecule Printing

Solutions of the 2011 library compounds (10 mM in DMSO) were arrayed onto the PALC glass slides with a high-precision microarrayer loaded with 16 microspotting pins. A map of the photo-cross-linked small-molecule microarrays is shown in the Supporting Information.The microarrayer was developed and customized at RIKEN. After arraying, the slides were allowed to stand on the platform typically overnight. The slides were exposed to UV irradiation of 4 J cm^{-2} at 365 nm by using a CL-1000L ultraviolet cross-linker (UVP Inc., CA, USA). They were then rinsed with DMSO, washed successively with DMSO, DMF, THF, DMSO, and Milli-Q water (1 h each) by using a MildMixer XR-36 instrument (TAITEC Co. Ltd., Saitama, Japan), and centrifuged (400*g*, 1 min). The slides were degassed and stored at −20 °C until use.

Binding Assay with Anti-Digoxin Antibody

Slide probing and washing procedures were performed at 25 °C with a Lucidea SlidePro hybridizer (Amersham Biosciences Corp., NJ, USA). In each experiment, the slides were incubated with a solution (0–20 μg mL^{-1}, 200 μL each) of a monoclonal mouse anti-digoxin antibody DI-22 clone (Sigma–Aldrich Inc., MO, USA) in binding buffer (1% skimmed milk, 10 mM Tris/HCl, 150 mM NaCl, 0.05% tween-20, pH 8.0) for 1 h and washed with 15 cycles of a continuous flow (20 μL s^{-1}, 1 min) and mixing (1 min) of TBS-T buffer (10 mM Tris/HCl, 150 mM NaCl, 0.05% tween-20, pH 8.0). The slides were then probed with a solution of a goat anti-mouse IgG–$Alexa_{633}$ conjugate (Invitrogen Co., CA, USA) (100 μg mL^{-1}) in binding buffer for 1 h and washed again with 15 cycles of a continuous flow (20 μL s^{-1}, 1 min) and mixing (1 min) of TBS-T buffer. The slides were rinsed with Milli-Q water (60 μL s^{-1}, 1 min), dried under a stream of air for 1 min, and centrifuged (400*g*, 1 min).

Competitive Binding Assay on Microarrays

Different amounts (final concentration: 0–1 μM) of digoxin in DMSO (final concentration of DMSO: 0.5%) were added to a solution of DI-22 monoclonal mouse anti-digoxin antibody (2 μg mL^{-1}) in binding buffer (325 μL). The mixtures were incubated at room temperature for 1 h and then used for slide probing. The procedures that followed were the same as described above.

Scanning Slides for Fluorescence

The probed slides were scanned at a resolution of 10 μm per pixel with a GenePix 4100 scanner (Amersham Biosciences) by using the Cy3 and Cy5 channels. The fluorescence signals were quantified with GenePix 5.0 software with local background correction. For quantitative analyses, two slides (i.e., four microarrays) were used for each experiment. The differences between the fluorescence signal intensity on the two slides were normalized by using rhodamine fluorescence at the Cy5 channel. Four normalized fluorescence signals were averaged and plotted.

Competitive Binding Assay with Dextran–Charcoal and [^{3}H]digoxin: Radioimmunoassay

Digitoxin, digoxigenin, and ouabain were purchased from Sigma–Aldrich Inc. Digoxin was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Other compounds were obtained from the Spectrum Collection library. DMSO (2 μL) or a solution (0.2 nM–20 μM) of a steroidal compound in DMSO was added to binding buffer (500 μL) with or without an anti-digoxin antibody (2.4 nM). The mixture was stirred under vortex for 10 s and incubated at 30 °C for 30 min. A solution (2 μL) of [^{3}H]digoxin (2 nCi, 0.17 nM) in EtOH was added to either mixture. The mixture was stirred under vortex for 10 s and incubated again at 30 °C for 1 h. During this period, dextran-coated charcoal (100 mg) was mixed with TBS-T buffer (10 mL) and rotated at 4 °C for 1 h. A portion (200 μL) of the resulting suspension of dextran-coated charcoal (1% *w*/*v*) was added to the mixture. The mixture was stirred under vortex, incubated at 4 °C for 10 min, and centrifuged (4 °C, 3000 rpm, 10 min). The separated supernatant (250 μL) containing antibody-bound [^{3}H]digoxin was added to scintillation fluid (3 mL; Aquazol-2, New England Nuclear, MA, USA), stirred under vortex for 10 s, and counted with a liquid scintillation counter. All assays were performed in triplicate.

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