Target Identification in Live Cells

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Discovery and Target Identification of an Antiproliferative Agent in Live Cells Using Fluorescence Difference in Two-Dimensional Gel Electrophoresis**

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Phenotype-based screening has been recognized as a key approach to the discovery of novel therapeutic agents because of the recent withdrawal of marketed drugs developed by conventional target-based medicinal chemistry.[1a] Furthermore, the identification of small molecules that control nonconventional drug targets has become increasingly important for curing diseases that are resistant to existing drugs, for the development of regenerative medicines, and for the treatment of incurable diseases.[1b,c] Phenotype-based assays facilitate the use of efficiency-based evaluations for the discovery of a small-molecule modulator for unknown drug targets, which leads to the development of novel classes of drugs or the discovery of new drugable protein targets. For this approach, it is essential to identify the mode of action of small-molecule modulators. However, understanding their mechanism, the target identification process in particular, is time-consuming, difficult to implement, and does not provide clear results. Therefore, there is a great demand for the development of new and robust methods for target identification.[2]

The conventional affinity-based pull-down method is hampered by nonspecific binding events owing to the large size of the probe and the use of solid beads. Also, the experimental buffer conditions can yield completely different patterns for the interactions between proteins and small molecules, which limits the robustness of this method.[3] Several research groups have addressed these issues by using photoaffinity activation and bioorthogonal transformation.[2g,4] Photoaffinity groups in bioactive small molecules can generate covalent linkages to adjacent target proteins upon irradiation, which can be suitable for target identification under various experimental conditions (for example, cell lysate labeling, live cell labeling, and ex vivo labeling) without being affected by buffers or salt concentrations. Bioorthogonal moieties allow the selective labeling of small-molecule-bound target proteins for visualization. Despite these advantages, nonspecific labeling on various proteins, especially on abundant and sticky proteins, as well as on actual target proteins, has been a major problem for the photoaffinity-based crosslinking method.[5] Furthermore, the differentiation of actual binding events from nonspecific binding is essential for the successful application of photoaffinity groups to target identification. To address this limitation, we have developed a new method called fluorescence difference in two-dimensional gel electrophoresis (FITGE), and employed it in the target identification of a new antitumor agent screened from our in-house small-molecule library.

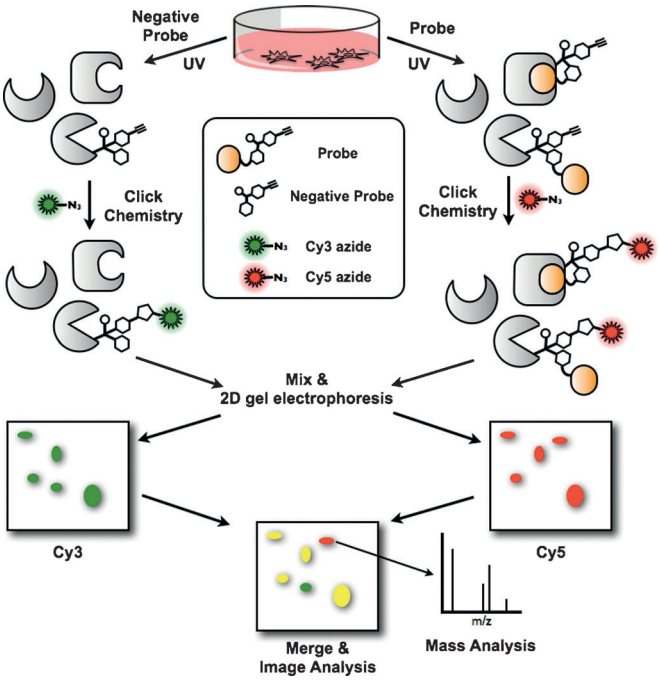

Figure 1. Outline of the FITGE method for identification of the targets of bioactive small molecules in live cells.

After identification of hit compounds from the cell-based phenotype assay and subsequent structure–activity relationship study, a bioactive small-molecule probe derived from a hit compound, and a corresponding negative probe can be prepared. Each probe bears a photoaffinity group and a bioorthogonal acetylene moiety to attach to and visualize binding events. As shown in Figure 1, the resulting probe and

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negative probe are separately incubated with live cells to induce specific binding events between proteins and small molecules. The subsequent UV irradiation of live cells allows the in situ generation of radical species on the photoaffinity group, which crosslinks the adjacent proteins with covalent bonds. After lysis of the treated cells, proteins labeled with probes can be visualized with fluorescent dyes using a bioorthogonal click reaction.[6] Owing to the high nonspecific labeling of photoaffinity groups,[5] the side by side comparison of labeling patterns between probe-labeled and negative-probe-labeled proteins is a critical step in the FITGE method; however, this comparison is difficult and misleading because of gel-to-gel inconsistency.[7] Researchers in the field of proteomics have addressed this issue by running two different samples with two different fluorescent dyes in a single gel in a procedure known as difference gel electrophoresis (DIGE).[8] Inspired by the DIGE strategy, probe-labeled and negative-probe-labeled proteomes are crosslinked to two different fluorescent dyes, pooled in a single sample, and separated by two-dimensional gel electrophoresis (2DGE). As probe-labeled and negative-probe-labeled proteomes are labeled with Cy5 and Cy3, respectively, nonspecific binding events can be ruled out by direct comparison of the labeling patterns of whole proteomes in 2DGE. Following fluorescent gel image analysis, the desired protein spots are cut out and subjected to mass analysis for the identification of potential target proteins.

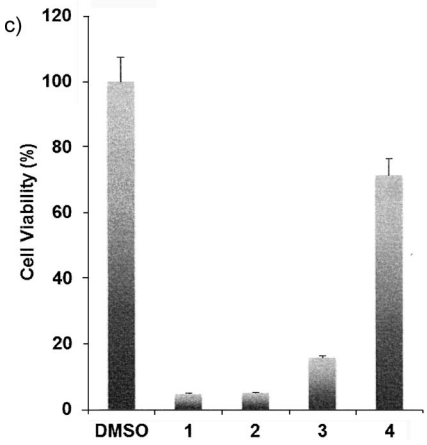
We also noticed that most studies in the field of target identification have been conducted using cell lysates, as these are more easily handled. However, the specific interactions between bioactive small molecules and target proteins in cell lysates can differ from those in live cells as a result of the extremely high concentration of proteins inside live cells, as well as the nonspecific attractive and repulsive interactions that occur in live cells with many macromolecules, including proteins, nucleotides, lipids, and metabolites.[9] Chang and coworkers reported the environment-dependent covalent labeling of an organic fluorophore to target proteins, but they did not focus on the functional modulation of target proteins.[10] Yao and co-workers successfully demonstrated the in vitro and in cell target identification of known bioactive small molecules.[11] Herein, we report the target identification of a novel bioactive small molecule discovered by phenotype-based screening using the FITGE method along with a systematic comparison of live cells with cell lysates. In particular, we aimed to demonstrate the importance of efficient high-resolution differentiation between specific and nonspecific binding.

We initiated probe design on the basis of an anticancer agent, which was identified using a cell-based proliferation assay against our 3000 member library of small molecules, constructed by privileged-substructure-based diversity-oriented synthesis (pDOS). The pDOS synthetic strategy employs the divergent recombination of polyheterocycles embedded with privileged substructures to maximize the molecular diversity of the drug-like small-molecule library.[12]

As shown in Figure 2a, compound **1** was antiproliferative toward HeLa (human cervical cancer cell line) with an IC_{50} of 450 nM, as well as U266 (human myeloma cell line), A549 (human lung cancer cell line), and MCF7 (human breast cancer cell line). Based on analogue activity patterns, we successfully introduced a long aliphatic chain at the C2 position of the benzopyran moiety and produced compound **2** without deterioration of biological activity (Figure 2c). This functional handle was used as the starting point of probe design for FITGE-based target identification. The desired probe **3** was synthesized with benzophenone and acetylene moieties at the C2 position of **1** to enable the temporal crosslinking of the target proteins upon UV irradiation and

a)

Cell line	IC_{50}
HeLa	(450 ± 4) nM
U266	(416 ± 21) nM
A549	(381 ± 13) nM
MCF7	(744 ± 32) nM



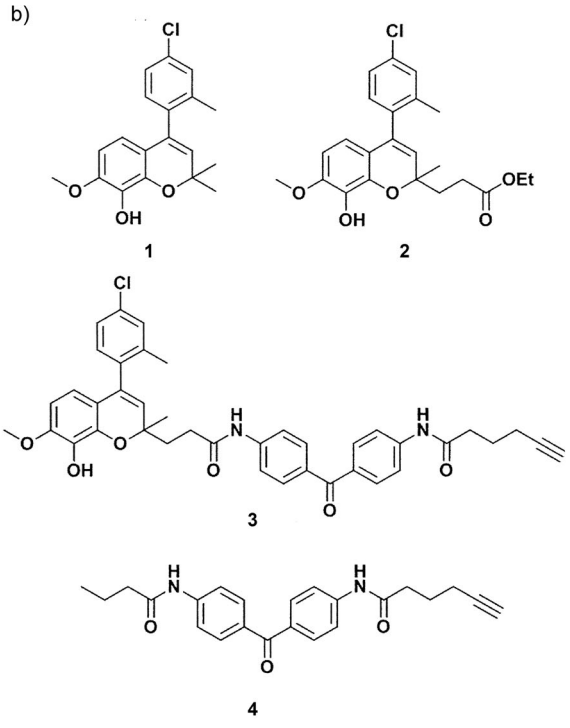


Figure 2. Biological activity of antiproliferative agents discovered by a pDOS chemical library screen. a) IC_{50}(± standard deviation) values of **1** in HeLa, U266, A549, and MCF7 cell lines. Each cell line was treated with **1** in various concentrations for 72 h. Cell cytotoxicity was measured with WST-1. b) Chemical structure of hit compound **1** and derivatives **2**–**4**, for target identification. c) Viability of HeLa cells after 72 h of treatment with compounds **1**–**4** (10 μM).

the fluorescence-based visualization of target proteins by a bioorthogonal click reaction, respectively (for a detailed procedure, see the Supporting Information). We also designed a negative probe **4** to eliminate nonspecific protein labeling. Prior to their application in target identification, the cellular activities of probe **3** and negative probe **4** were tested. As shown in Figure 2c, we confirmed that probe **3** still has antiproliferative activity, but negative probe **4** does not; therefore, these probes possess the essential criteria for use in target identification.

Probe **3** and negative probe **4** were incubated separately with cell lysates and live cells. During incubation, UV irradiation at 365 nm was used to induce covalent crosslinking of probes with adjacent proteins. Proteomes from each condition were then modified with a Cy5–azide fluorescent label by a click reaction and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). However, we did not observe any significant difference in bands based on the fluorescence labeling pattern (Figure 3a). We suspected that the amount of target proteins was too small to distinguish changes in bands by one-dimensional gel electrophoresis (1DGE). Therefore, we enriched the target proteins using a traditional pull-down method in conjunction with UV-induced crosslinking. Proteomes covalently linked to **3** and **4** were labeled with a biotin–azide linker by a click reaction, after which the labeled proteins were enriched by an affinity pull-down method using a streptavidin bead. The enriched proteomes were eluted, separated by 1DGE, and visualized by silver staining. Interestingly, we observed different protein patterns after enrichment, and a single labeled protein band (Figure 3b, indicated by an arrow) in the cell lysate appeared to be the target protein of antiproliferative agent **1**, which was identified as heat shock protein 60 (HSP 60) by MS analysis. For target validation, we performed a malate dehydrogenase (MDH) refolding assay to measure the enzymatic activity of HSP60.[4a] As shown in Figure 3c, MDH refolding by HSP60 was inhibited by **1** at 10 μM, which is similar to epolactaene *tert*-butyl ester (ETB), a known HSP60 inhibitor. However, the drastic concentration-dependent reduction of the inhibition activity of **1** on HSP60, even at 5 μM, suggested that HSP60 cannot be a major target protein of **1**, which exhibits excellent antiproliferative activity with a submicromolar IC_{50} value.

Undaunted by a series of failures in target identification, we pursued the possibility that the analysis of the entire proteome display using 2DGE could address the poor resolution of 1DGE. To address the intrinsically high nonspecific labeling obtained when using the benzophenone moiety as a photocrosslinker, the proteomes labeled with probe **3** and negative probe **4** were also visualized with Cy5 and Cy3, respectively. To compare the images from two fluorescence channels and eliminate the nonspecifically labeled proteins, proteomes labeled with **3** and Cy5 or **4** and Cy3 were subjected to a single round of 2DGE and the merged images of the Cy5 and Cy3 channels were then analyzed. The merged image revealed three fluorescent colors, red and green spots from proteins preferentially labeled with probe **3** and negative probe **4**, respectively, and yellow spots from proteins dual-labeled with **3** and **4**. Surprisingly, we also clearly observed some red spots among a large collection of green and yellow spots, which we ruled out as proteins labeled nonspecifically by negative probe **4** (Figure 4). We believe that the higher resolution obtained by 2DGE over 1DGE enables the discrimination of the actual target proteins from nonspecifically labeled proteins.

Furthermore, the labeling pattern using the FITGE method in cell lysates was different from that in live cells. This finding demonstrates the importance of the environment during target identification. As shown in Figure 4a, FITGE-based labeling under cell lysate conditions revealed a single red spot c that was selectively labeled by **3**, but not by **4**. The merged image showed the proteins dual-labeled by **3** and **4** in yellow, such as spots a and b, which helps to deprioritize them for further mass analysis. Based on this labeling pattern in cell lysate, red spot c represented a strong target protein candidate. Subsequent mass analysis revealed that this spot was HSP60, which was identical to the results obtained using our previous pull-down enrichment. As HSP60 is not a major target protein of **1** and was confirmed to be inhibited by compound **1** only at high concentrations, we further investigated the FITGE method in live cells. As shown in Figure 4b, negative probe **4** labels more proteins in live cells than in the cell lysate, which is probably a result of the high

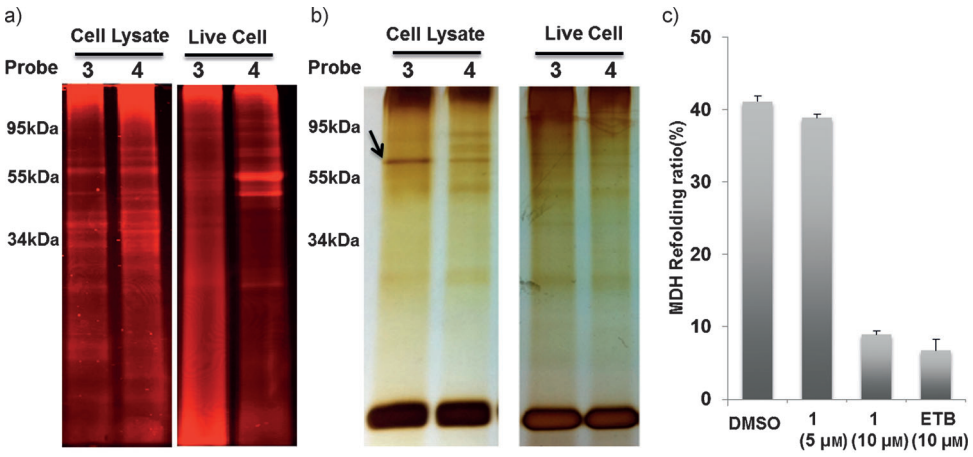


Figure 3. Target identification and validation. a) **3** and **4** were separately covalently linked to proteomes from cell lysates and live cells. The labeled proteomes were visualized with Cy5–azide and analyzed by 1DGE. b) The labeled proteomes were covalently linked with biotin–azide, enriched by affinity pull-down, separated by 1DGE, and visualized by silver staining. The arrow indicates a potential target protein. c) Malate dehydrogenase (MDH) refolding assay. Epolactaene *tert*-butyl ester (ETB) is a known inhibitor of MDH refolding by HSP60.

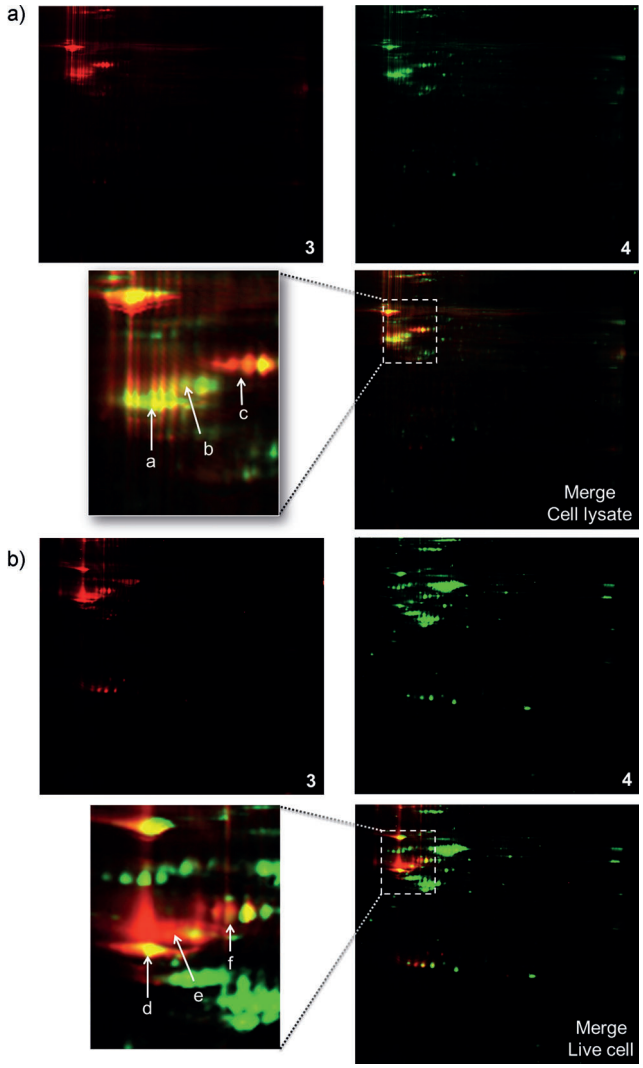


Figure 4. Target identification using the FITGE method. Cy5 channel image (proteome labeled with **3**) and Cy3 channel image (proteome labeled with **4**) are merged into one image for analysis. a) Cell lysate proteome labeling image. b) Live cell proteome labeling image.

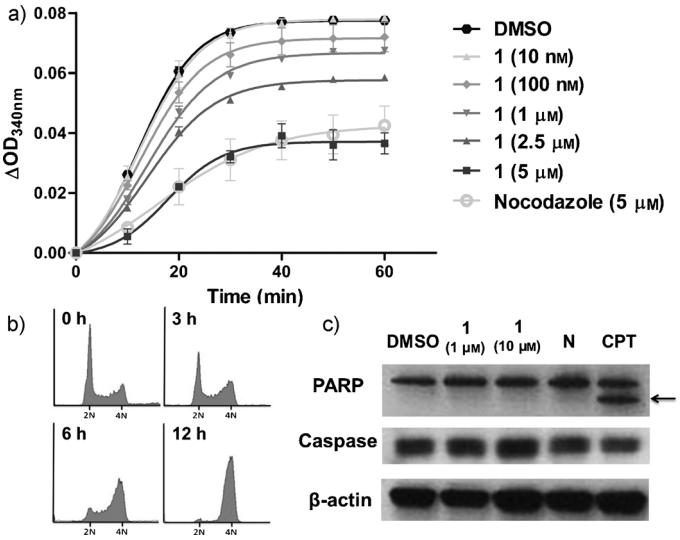


Figure 5. Validation of tubulin as a target protein of **1**. a) An in vitro tubulin polymerization assay showing dose-dependent inhibition by **1**. ΔOD_{340nm} = optical density difference at 340 nm b) Flow cytometry cell cycle analysis of HeLa cells treated with **1** (1 μM). c) Western blot analysis of the apoptotic pathway. N = nocodazole (10 μM), CPT = camptothecin (10 μM). The arrow indicates cleaved PARP.

protein concentration in live cells. The number of spots labeled by probe **3** was similar under both conditions, but the size of individual red spots was slightly different. In live cells, spots e and f were preferentially labeled by **3**, but not by **4**. Interestingly, spots d, e, and f are similar to spots a, b, and c in terms of molecular weight and isoelectric point (pI) value, but their labeling patterns are different; unlike spots a and d or spots c and f, which are consistently labeled in yellow or red, respectively, spots b and e were labeled in red in live cells, but labeled in yellow in the cell lysate. Subsequent MS analysis revealed that spot f is HSP60, but spot e is tubulin, which can be a potential target protein of **1**. These results suggest that the FITGE method allows significantly simplified protein mass analysis, as compared to the 1D pull-down enrichment method, through sensitive detection in the labeling preference of active and negative probes, and thus provides a potential solution to overcome the current limitation of extensive nonspecific binding of photoaffinity probes.

Target validation was then performed using an in vitro tubulin polymerization assay. As shown in Figure 5a, compound **1** inhibited tubulin polymerization in a dose-dependent manner and showed a similar activity to nocodazole,[13] a known tubulin polymerization inhibitor. Even at 100 nM, compound **1** still showed some inhibition of tubulin polymerization. Considering the antiproliferative activity of **1**, this result strongly supports tubulin as the target protein of **1**. As the hallmark of cellular response toward tubulin polymerization inhibitors is a cell-cycle arrest,[14] we tested whether **1** triggers cell-cycle arrest using flow cytometry. As shown in Figure 5b, the cell cycle of HeLa cells was arrested upon treatment with **1** at 1 μM in a time-dependent manner, and the cell cycle completely stopped after 12 h. However, western blot analysis showed that neither caspase 3 nor poly(ADP–ribose)polymerase (PARP) were activated after 12 h of treatment with **1**, which is similar to nocodazole. Instead, a different type of anticancer agent, camptothecin (a DNA topoisomerase I inhibitor),[15] triggered the apoptotic pathway (Figure 5c). We also confirmed the inhibition of tubulin polymerization upon treatment of **1** by cell-based fluorescent imaging through the visualization of tubulin and its microtubules (Supporting Information, Figure S1–2). Therefore, we have concluded that **1** is a potent antitumor agent that functions by the inhibition of tubulin polymerization.

In conclusion, we have developed a new target identification method, FITGE, which aims to observe interactions between proteins and small molecules in an intact cellular environment. After a series of failures using conventional target identification methods, we successfully identified the protein target of anti-proliferative compound **1** with FITGE only in live cells, and observed the environment-dependent binding events of a functional small molecule by direct comparison between live cells and cell lysates. Even though it

still requires the synthesis of bioactive probes with a photocrosslinker moiety, the FITGE method can address the current limitations of conventional target identification methods and can significantly enhance the possibility of target identification through the combination of the covalent capturing of target proteins in an intact cellular environment and the efficient exclusion of nonspecific protein labeling using two-color 2DGE. We believe our FITGE method provides a unique means of target identification in live cells.

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