

**Novel methods for drug discovery and development
using ligand-directed chemistry**

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Preface and Acknowledgements

The studies presented in this dissertation have been carried out under the direction of Professor Itaru Hamachi at the Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, from April 2012 to September 2016. The study is focused on novel methods for drug discovery and development using ligand-directed chemistry.

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General Introduction

Introduction

Chemical drugs affect the processes of the human mind or body. It is also known that most drugs currently used for human therapy interact with proteins, altering their activity. Drug development is the process of bringing a new pharmaceutical drug to the market once a lead compound has been identified through the process of drug discovery (**Fig. 1**). The process usually takes up to 10 to 17 years from hit identification to clinical approval, with estimated costs of up to 1 billion US dollars. Moreover, R&D productivity for drug development has been recently declining for a number of years^{1,2}.

Over recent years, the application of chemical biology has become highlighted within drug discovery and development in order to enhance overall R&D productivity³. Its application for drug discovery have been required to develop efficient screening methods that find novel chemotypes, have superior selectivity profiles, or show less artifacts^{4,5}. The application of chemical biology for drug development have been expected to develop powerful methods that monitor efficacious drug concentration, identify off-target of drug in cells³.

In the chapter, I summarize current methods for drug discovery and development relied on techniques of chemical biology.

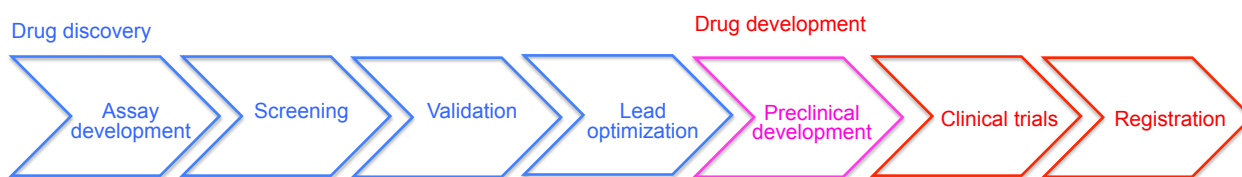


Fig. 1 Workflow for drug discovery and development.

Genetic methods for drug discovery and development

The recently advanced genetic manipulation method enables us to fuse a target protein with various functional protein or peptide, which makes the chimera protein on/in cell or tissue. This technique is widely applied to the research field of chemical biology.

A method for drug development using SNAP tag

Johnsson and co-workers developed a covalent protein labeling system, called SNAP tag, using human O6-alkylguanine transefrase (hAGT) as a protein tag^{6,7}. hAGT, which naturally dealkylates O6-alkylated guanine residues in damaged DNA by alkylation of a cysteine residue of its the active site, can react with fluorescent benzyguanine derivatives (**Fig. 2a**).

For many drugs, finding the balance between efficacy and toxicity requires monitoring their concentrations in the patient's blood. They also have developed semisynthetic bioluminescent sensors using SNAP tag, that permit precise measurements of drug concentrations in patient samples⁸. The bioluminescent sensors are ratiometric, and are made up of three components: a receptor protein for the drug of interest, a luciferase and a synthetic molecule containing a fluorophore and a ligand for the receptor protein (**Fig. 2b**). The attached ligand binds to the receptor protein in an intramolecular manner, bringing the fluorophore close to the luciferase and permitting efficient bioluminescent resonance energy transfer (BRET)⁹. Sufficient concentrations of analyte can displace the ligand from the receptor protein, causing the BRET efficiency to decrease (**Fig. 2b,c**). The biosensor was amenable to observe a methotrexate-dependent color transition from red to blue not only in serum but also in whole blood (**Fig. 2d**), and additionally sample analysis could be achieved with simple digital camera.

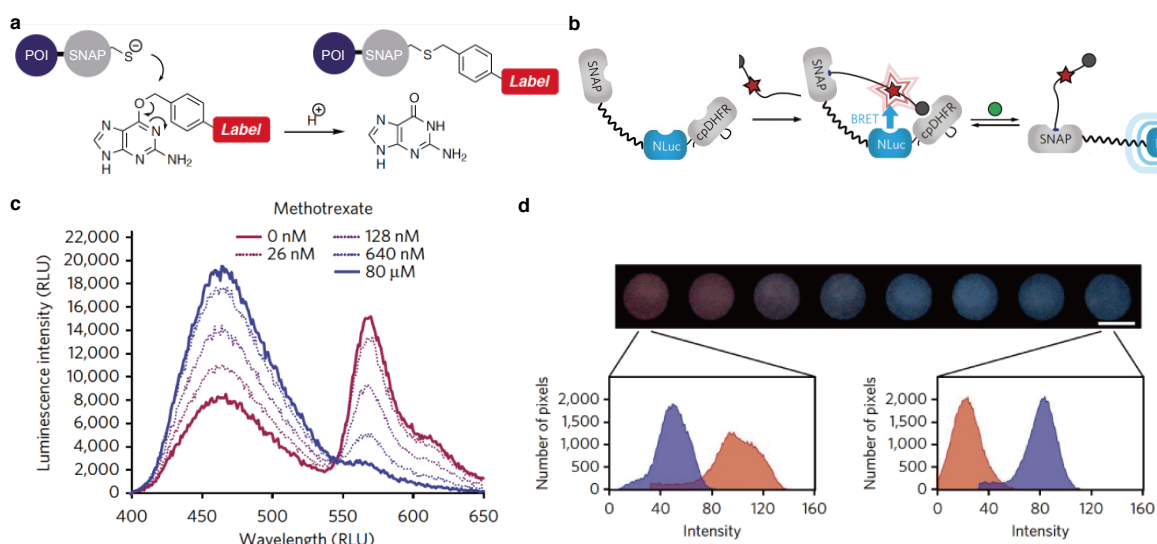


Fig. 2 (a) Schematic illustration of covalent labeling using SNAP tag. (b) Schematic illustration for the construction of bioluminescent sensor. Fluorophore, DHFR inhibitor, free analyte are shown as red star, gray ball, and green ball, respectively. (c) Emission spectra of SNAP tag fused DHFR labeled with a fluorophore by the addition of methotrexate (analyte). (d) Picture of SNAP tag fused DHFR labeled with a fluorophore with varying methotrexate concentrations in human serum taken with a digital camera.

A method for drug discovery using site-directed mutagenesis

Site-directed mutagenesis is a technique for studying the structure and function of protein of interest. The site-specific replacement with cysteine is the most widely conducted approach to covalently couple a synthetic fluorophore to a protein of interest, and cysteine-mutated proteins can be easily labeled with maleimide, enone, or iodoacetamide group (**Fig. 3a**)¹⁰.

Targeting allosteric site of kinases is thought to be a promising strategy for overcoming bottlenecks in kinase inhibitor research, such as limited selectivity and drug resistance. Rauh and co-workers developed a screening assay for allosteric inhibitors of the tyrosine kinase cSrc using site-directed mutagenesis^{11,12}. In this method, a cysteine was mutated into the activation loop of cSrc for subsequent labeling with the environmentally sensitive fluorophore acrylodan (**Fig. 3b**). The activation (DFG) loop is a crucial regulator of the substrate binding cleft. The equilibrium between the DFG-in and DFG-out conformations can be modulated by phosphorylation, by protein-protein interactions and by the binding of different inhibitor classes (type I, II or III inhibitors) (**Fig. 3b,c**) in many kinases¹³, and conformation of active loop were significantly different among the each conformation. Owing to this property, the binding of inhibitors that favor either the DFG-in or DFG-out conformation induced changes in the environment of the fluorophore and altered its fluorescence properties (**Fig. 3d**). They used this acrylodan-modified cSrc to screen a library of ~35000 small organic molecules, and hit molecules which can be developed into promising new lead compounds was identified¹⁴.

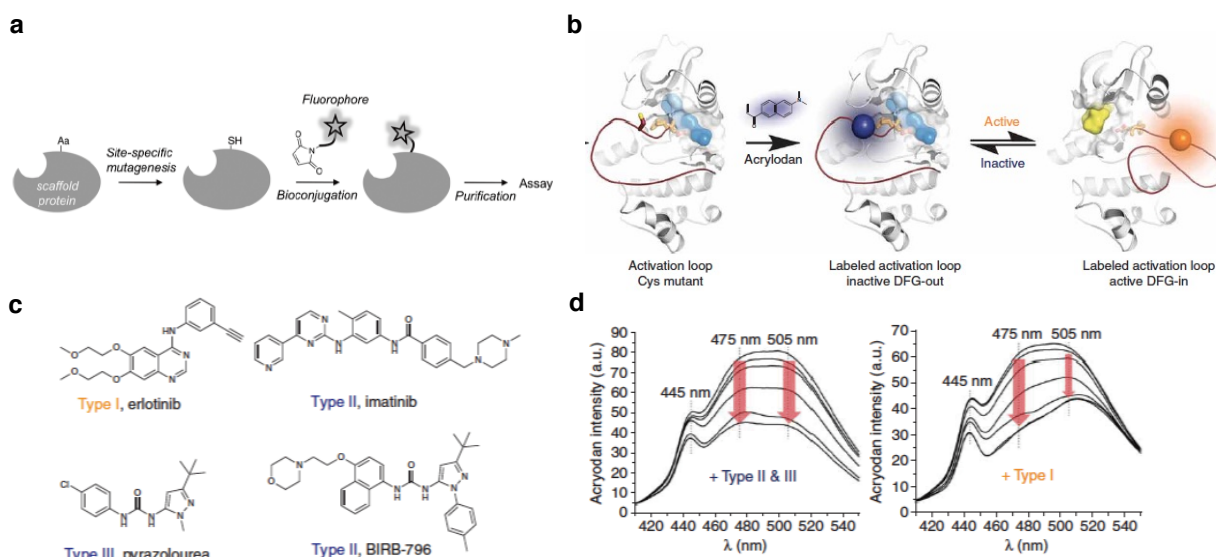


Fig. 3 (a) Schematic illustration of site-directed mutagenesis for subsequent labeling with a fluorophore. (b) Structural model of a protein kinase of interest with the DFG motif (orange) and activation loop (red) highlighted. (c) Examples of type I, type II and type III kinase inhibitors and scaffolds. (d) In the absence of ligand, acrylodan-labeled cSrc shows two emission maxima at 475 nm and 505 nm.

A method for drug discovery using LOV (light-oxygen-voltage) domain

LOV domains bind flavins as prosthetic groups and act as reversible light sensors in bacteria, fungi and plants (**Fig. 4a**)¹⁵. LOV domains control heterogeneous effector domains such as enzymes or transcriptional regulators. Dimerization of LOV domains was proposed to play an important role in effector domain regulation.

Janovjak and co-workers have demonstrated that, in the screening method, the use of light for both activation and detection of cell signaling with LOV domain fused receptor tyrosine kinases (Opto-RTKs)^{16,17}. Opto-RTKs are modified growth factor receptors that are insensitive to their natural ligands but activated by blue light–induced homodimerization through incorporation of the LOV domain (**Fig. 4b**). In the fluorescent reporter, SRE-GFP, tandem repeats of serum response element (SRE), an enhancer sequence responsive to signaling pathways including the MAPK/ERK pathway, are placed upstream of a gene coding for a green fluorescent protein (GFP) (**Fig. 4c**). They proceeded to screen a kinase inhibitor library against the light-activated fibroblast growth factor receptor 1 (Opto-FGFR1) and identified the inhibitors which interact with FGFR1¹⁶. Because peptides and other agonists are not required, the method can also screen against ‘orphan’ receptors—those for which native ligands are not known.

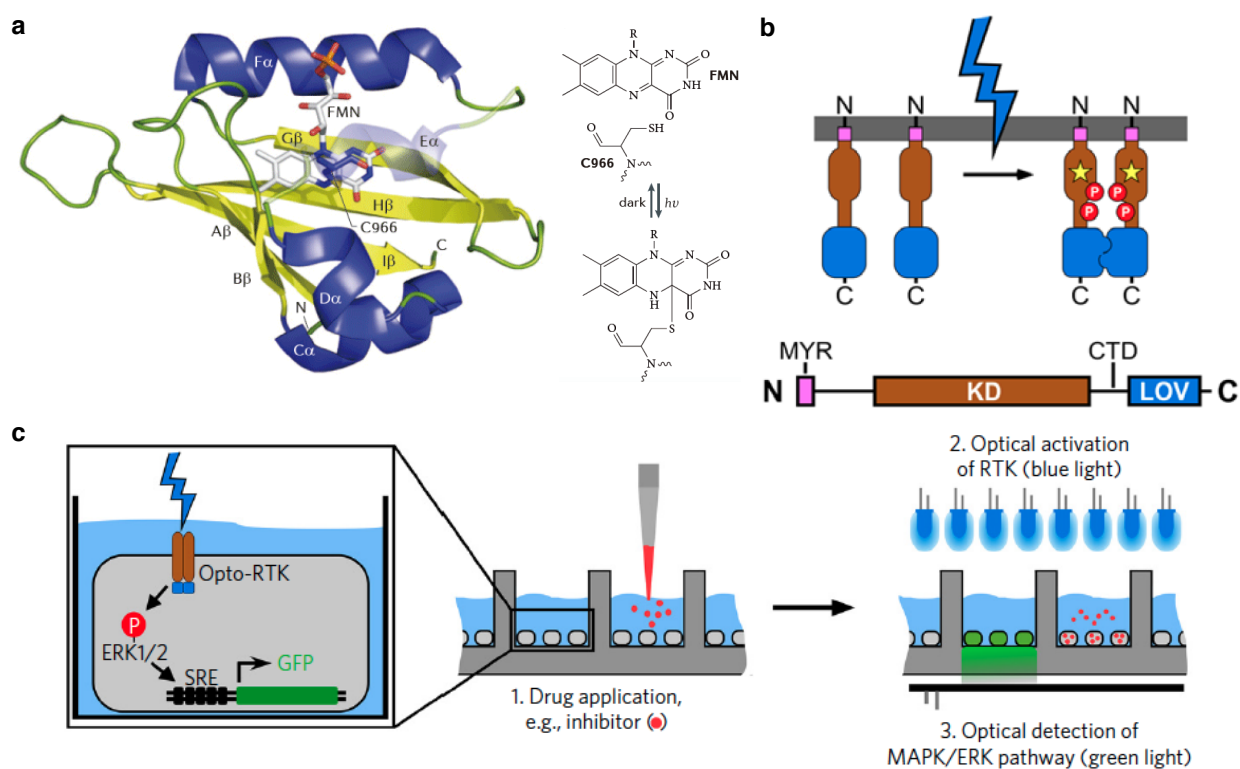


Fig. 4 (a) Crystal structure of the LOV domain (Protein Data Bank accession 1JNU). (b) Schematic illustration of Opto-RTK activated by blue light. (c) Cell-based optical screen method against RTKs and the MAPK/ERK pathway.

Non-genetic methods for drug discovery and development

As mentioned above, the technique relied on genetic manipulation is valuable for drug discovery and development, notably construction of the biosensor. Next, I describe that techniques of chemical biology without performing gene manipulations are also useful for drug discovery and development under physiological conditions.

A method for drug discovery using ABPP

Activity-based protein profiling (ABPP) is a chemical proteomic technology developed by Cravatt and co-workers^{18,19}. This method makes use of reactive chemical probes to covalently modify enzyme active sites in native biological setting (**Fig. 5a**). When performed in a competitive format, where compounds are assayed for their ability to block probe labeling, ABPP offers a powerful platform to discover small-molecule inhibitors of enzymes (**Fig. 5b**)²⁰.

They employed this ABPP probe to perform a enzyme library-versus- small molecules library competitive ABPP screen against the mammalian serine hydrolase (SH) superfamily²¹. In this screening, labeled protein was analyzed by SDS-PAGE, which was followed by quantifying the fluorescence intensity of the protein bands on the gel, relative to a control (DMSO-treated) proteome (**Fig. 5c**). From this screening process, lead inhibitors were discovered for more than 30 SHs (**Fig. 5d**). Notably, several of these carbamates were found to selectively inactivate a single SH.

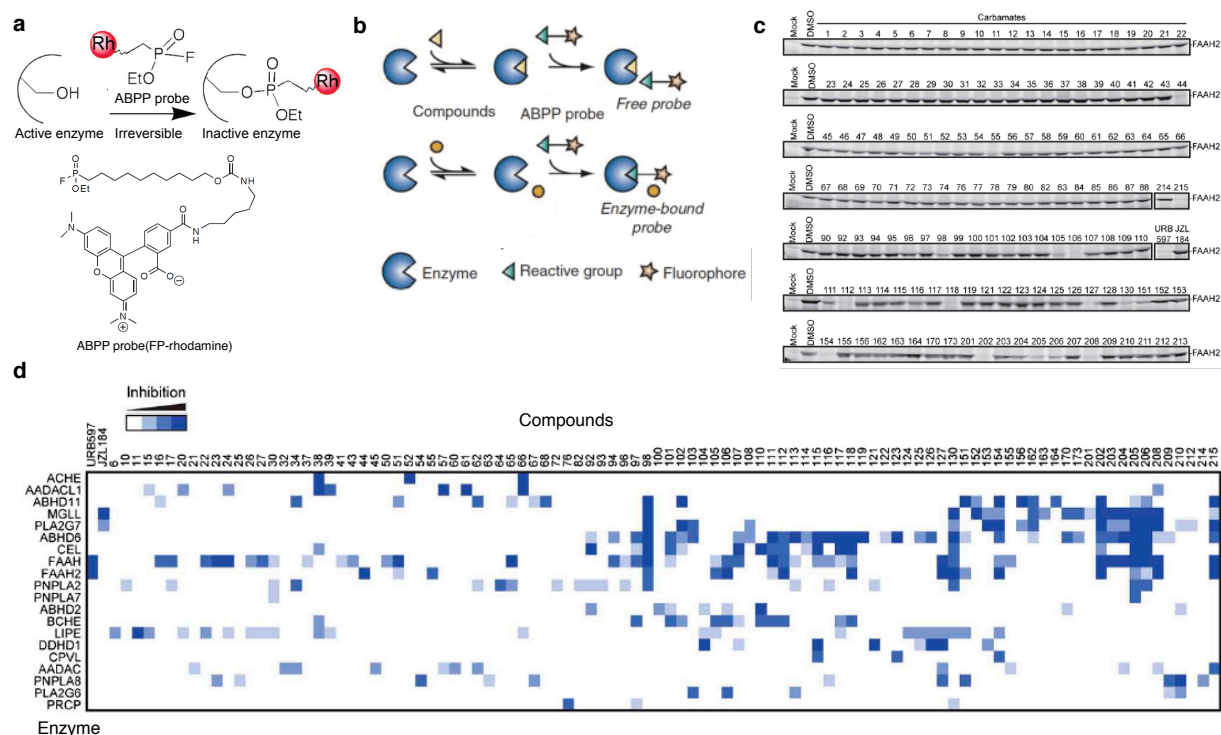


Fig. 5 (a) General mechanism for irreversible inhibition of serine hydrolases by rhodamine-tagged fluorophosphonate (FP) reagents. (b) Schematic illustration of the competitive ABPP. (c) Representative example of the primary competitive ABPP screening data for the enzyme FAAH2. (d) Hierarchical cluster analysis of carbamate inhibition profiles for a representative subset of SHs.

A method for drug development using irreversible drug

Kinases are principal components of signal transduction pathways and the focus of intense basic and drug discovery research. Irreversible kinase inhibitors that form covalent bonds with cysteine in the ATP-binding pocket of kinase have a number of potential advantages including prolonged pharmacodynamics, suitability for rational design, high potency²². Thus, a part of the inhibitors have emerged as valuable probes and approved drugs. However, many protein classes have functional cysteines, and therefore understanding the proteome-wide selectivity of covalent kinase inhibitors is imperative.

Cravatt and co-workers accomplished this objective using ABPP coupled with quantitative MS analysis in human cells²³. ABPP experiments were performed in cancer cells using clickable probe bearing irreversible inhibitor (ibrutinib), that is approved. Probe-labeled proteins were visualized by SDS-PAGE, indicating that clickable probes can detect ‘on’- and ‘off’-targets for drug (**Fig. 6a**). Most of the off-targets was identified by ABPP-SILAC, and it was revealed that the off-target protein have active cysteine residues (**Fig. 6b,c**).

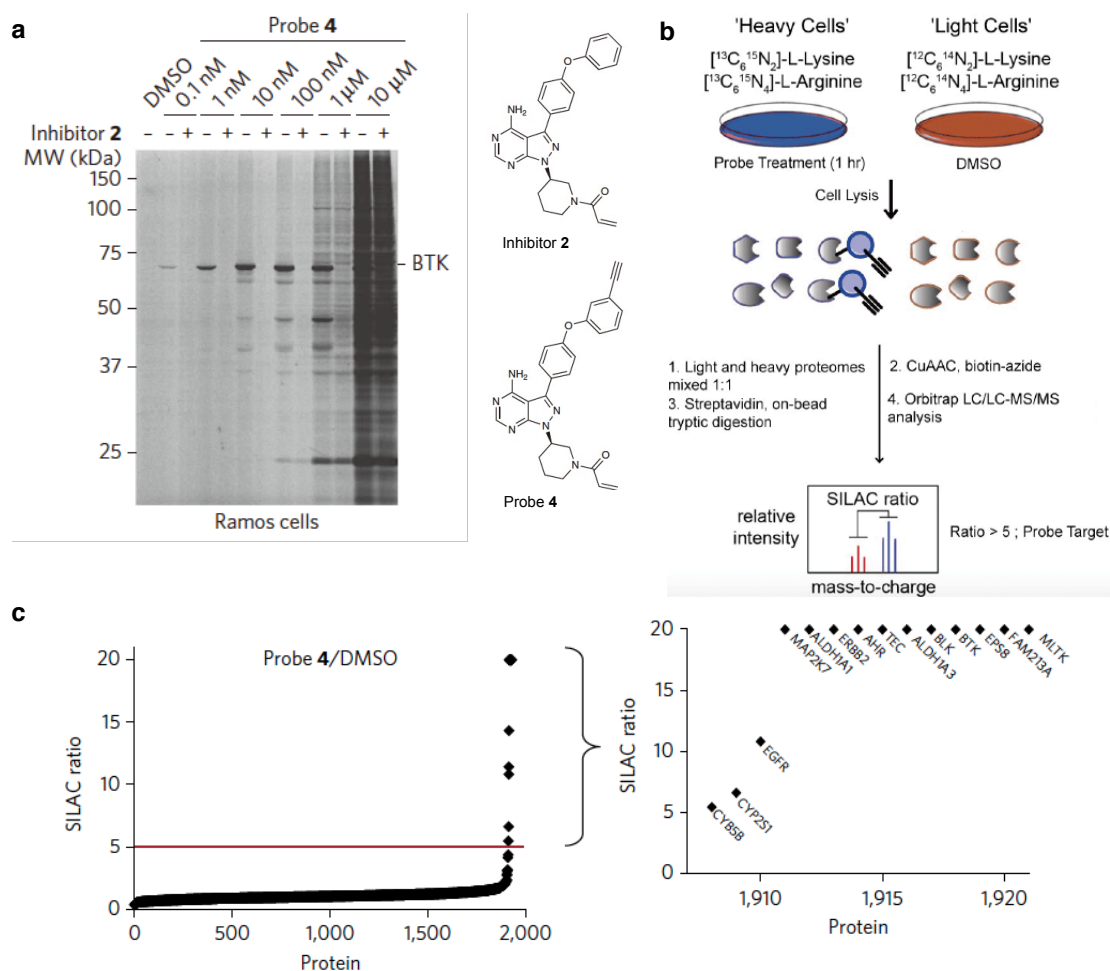


Fig. 6 (a) On (Bruton's tyrosine kinase, BTK)- and off-targets labeling using clickable probe bearing irreversible inhibitor in cancer cells. Inhibitor 2 was ibrutinib. (b) Workflow for SILAC-ABPP studies. (c) SILAC ratio plots for total proteins identified in experiments comparing cells treated with probe 4 versus DMSO (no probe).

Ligand-directed chemistry as a technique of chemical biology

As mentioned above, the labeled protein was inactivated by ABPP or irreversible drug, and ligand binding pocket of the protein was blocked by the probe or the drug. Therefore, these methods are not able to construct of the biosensor for the ligand binding pocket.

Recently, Hamachi group achieved specific endogenous protein labeling *in vivo* or on cell using novel labeling strategies, termed ligand-directed tosyl (LDT) chemistry^{24,25} and ligand-directed acyl imidazole (LDAI) chemistry^{26,27} (**Fig. 7a,b**). The S_N2-type reaction or acyl transfer reaction between the reactive group and a nucleophilic amino acid residue result in the release of the ligand moiety upon the labeling reaction. Therefore, the protein function can be recovered after washing out the excess labeling reagents and the cleaved ligand moieties. These approaches were applied not only for chemically labeling proteins in live cells or living mice, but also for constructing a protein-based biosensor without genetic engineering (**Fig. 7c,d**).

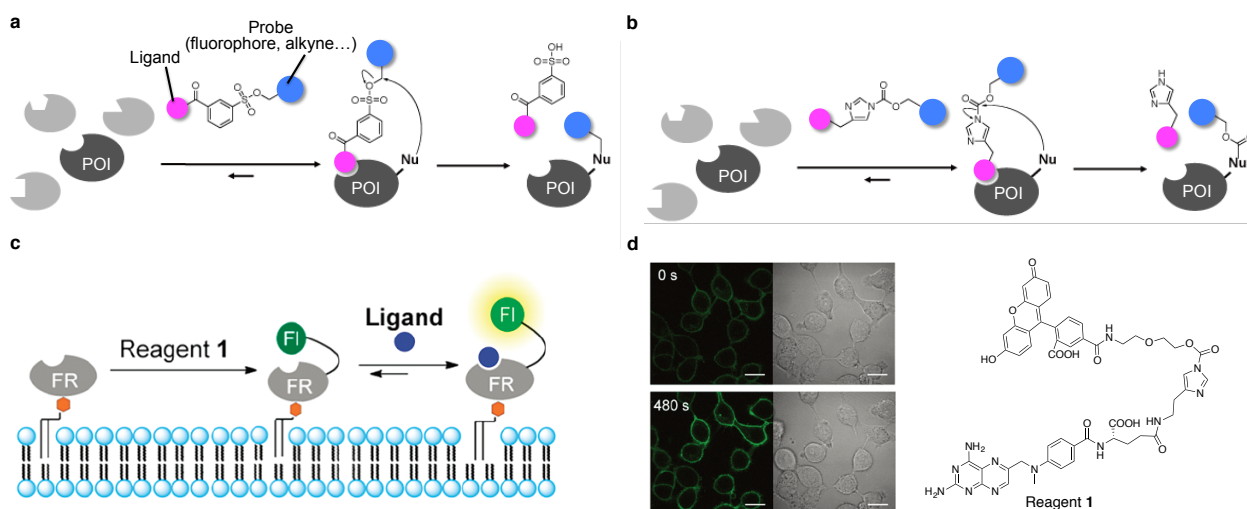


Fig. 7 (a) Schematic illustrations of target protein labeling by LDT chemistry. POI indicates protein of interest. (b) Schematic illustrations of target protein labeling by LDAI chemistry. (c) Construction and fluorescence sensing of FR-based biosensor on live cells. (d) Fluorescence imaging of a folate receptor on cell surface of live KB cells labeled with reagent 1 in the absence (0 s) or presence (480 s) of Folate.

Summary of this thesis

As described above, many researchers have developed various methods for drug discovery and development. However, the application of chemical biology has been still yet insufficiently done for drug discovery and development. Therefore, I have developed novel methods for drug discovery and development on the basis of ligand-directed chemistry. In this thesis, I established a novel drug assay method for a neurotransmitter receptor, GABA_AR (chapter 1), and utilizing its assay method to screen a library to discovery new allosteric small molecule modulators for GABA_AR (chapter 2). I also identified off-target of lapatinib using ligand-directed tosyl chemistry (chapter 3) (**Fig. 8**).

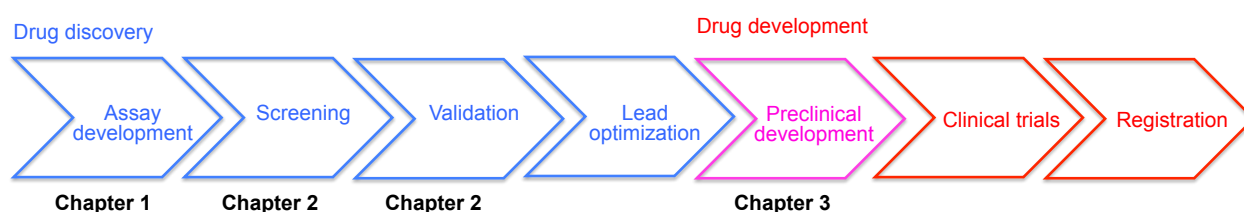


Fig. 8 Workflow in this thesis

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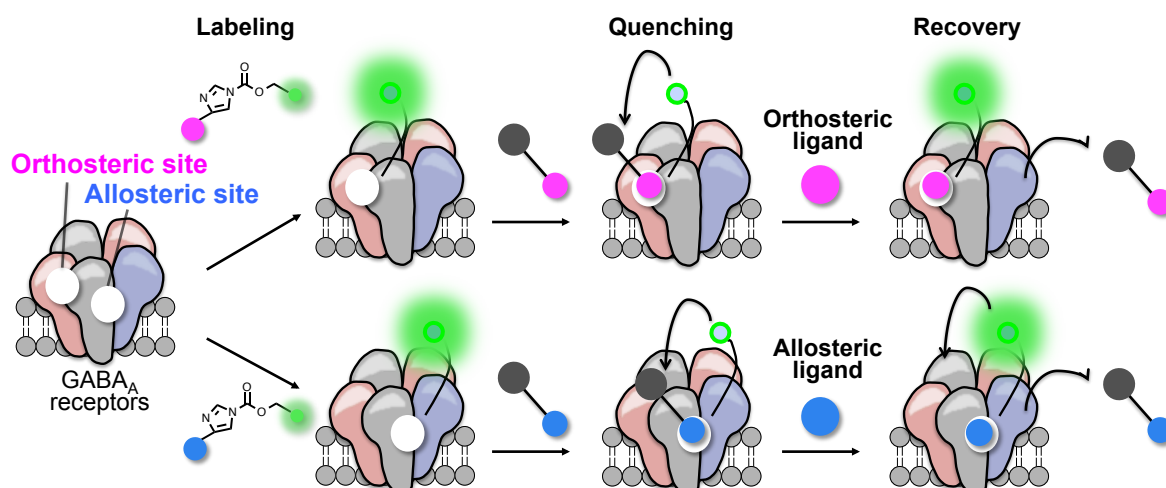
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Chapter 1

Construction of protein-based biosensor using ligand-directed chemistry for detection of ligand binding event

Abstract

The fast inhibitory actions of γ -aminobutyric acid (GABA) are mainly mediated by GABA_A receptors (GABA_ARs) in the brain. The existence of multiple ligand-binding sites and a lack of structural information have hampered the efficient screening of drugs capable of acting on GABA_ARs. I have developed semisynthetic biosensors for orthosteric and allosteric GABA_AR ligands on live cells via the coupling of affinity-based chemical labeling to a bimolecular fluorescence quenching and recovery system. By this method, biosensors composed of $\alpha 1$ -, $\alpha 3$ -, or $\alpha 5$ -containing GABA_ARs for benzodiazepine-site ligands can be flexibly constructed, and $\alpha 1$ - or $\alpha 5$ -subtype selective drug was successfully distinguished using these sensors.



1-1. Introduction

γ -Aminobutyric acid (GABA) is the major inhibitory neurotransmitter, whose actions are regulated upon binding to ionotropic GABA_A or metabotropic GABA_B receptors. The fast inhibitory actions of GABA are mainly mediated by GABA_A receptors (GABA_ARs), making it an invaluable pharmacological target^{1,2}. GABA_ARs assemble into a functionally active pentamer consisting of a variety of subunits to form GABA-gated chloride channels (**Fig. 1**). Nineteen different subunits ($\alpha 1$ - $\alpha 6$, $\beta 1$ - $\beta 3$, $\gamma 1$ - $\gamma 3$, δ , ϵ , π , θ , and $\rho 1$ - $\rho 3$) have been identified in GABA_ARs, which give rise to a remarkable level of diversity and complexity (more than 800 different pentamers). The current consensus describes that at least 20-30 isoforms may be involved in the brain and GABA_ARs consisting of different combinations of these subunits exhibit distinct pharmacological responses³. It is noteworthy, however, that most physiologically significant heteromeric GABA_AR formats are composed of two α subunits, two β subunits, and one γ subunit.

In addition to small molecules capable of interacting with the orthosteric GABA-recognition site, a wide variety of different allosteric modulators for GABA_ARs have also been reported (**Fig. 1**). Among them, benzodiazepine derivatives are well known to be potent for their sedative/hypnotic and anxiolytic effects⁴. Barbiturates, etomidate, propofol, and neurosteroids, which are all widely used as anesthetics, can also function as allosteric modulators, despite the fact that they have different interaction sites from that of benzodiazepines⁵. It is now generally accepted that the medicinal effects of these small molecules are largely dependent on the nature of their interaction site on the GABA_AR. The development of potent modulators capable of acting on specific allosteric sites is therefore critical for supporting drug discovery efforts for GABA_ARs.

Fluorescent semisynthetic biosensors have attracted considerable interest in recent years as chemical tools for drug discovery, which can be applied to both biochemical- and cell-based high-throughput screening platforms⁶⁻¹¹. Semisynthetic biosensors can be prepared using a site-specific chemical modification strategy with a synthetic fluorescent dye. For instance, Rauh and co-workers established a new biochemical screening system capable of identifying kinase^{12,13}, phosphatase¹⁴, and estrogen receptor inhibitors¹⁵. With regards to the construction of biosensors on live cells, Johnsson and co-workers recently designed a new class of semisynthetic biosensors termed SNAP-tag based indicator with a fluorescent intramolecular tether (Snifits) using an elaborate protein tag-based labeling technology coupled with Förster resonance energy transfer (FRET)¹⁶⁻¹⁸. Although undoubtedly powerful, these approaches require structural or amino acid sequence information pertaining to the ligand-binding domain. For GABA_ARs, affinity labeling, point mutagenesis, homology model study, and crystal structure of homomeric $\beta 3$ -subunit provided indirect informations on the putative ligand binding domains⁵. Still, structural analyses of naturally functional heteromeric GABA_ARs with atomic resolutions have not been completed, which represents a significant obstacle to the rational design of semisynthetic biosensors using these techniques.

Affinity-based protein labeling techniques¹⁹⁻²⁴, including ligand-directed chemistry²⁵⁻²⁸, can be used as effective alternatives for the protein-selective and site-specific modification of proteins with synthetic probes. Notably, these techniques do not require structural or sequence information data in most cases. Herein, I report the development of affinity-based GABA_AR labeling reagents that can covalently label a fluorophore in the vicinity of the targeted ligand-binding sites without having an adverse impact on the receptor functions. By coupling this chemical labeling with a bimolecular fluorescence quenching and recovery (BFQR) system, I have successfully constructed semisynthetic biosensors for various GABA_AR ligands on live cell surfaces. Taking advantage of affinity-based labeling, these two types of “turn-on” fluorescent biosensors are capable of detecting small molecules bound to the allosteric benzodiazepine site, as well as the orthosteric GABA site.

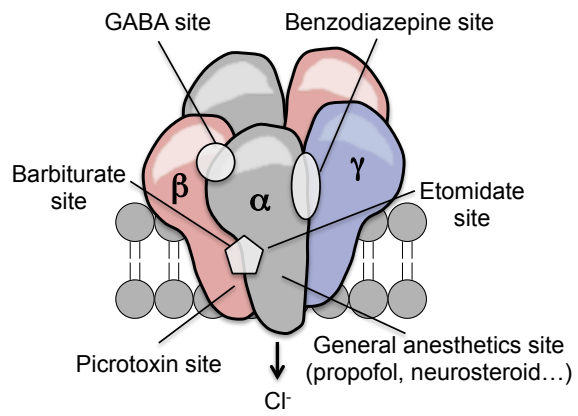


Fig. 1 Structural illustration of GABA_ARs and associated ligand-binding sites.

1-2. Results and Discussions

1-2-1. Design of semisynthetic biosensors for GABA_AR ligands.

I sought to use the BFQR system to construct GABA_AR-based semisynthetic biosensors for a diversity of GABA_AR ligands with a “turn-on” mode on live cells (**Fig. 2a**). For my strategy, it was envisaged that a fluorophore was covalently attached in the vicinity of the ligand-binding site without having an adverse effect on the receptor functions. This fluorescence can then be efficiently quenched upon the juxtaposition of a quencher to the tethered fluorophore, which is achieved through the conjugation of the quencher molecule to the ligand. The quencher-ligand conjugate bound to the ligand-binding site is then competitively replaced with small molecules having a sufficient affinity for the same binding site, which results in the recovery of the fluorescence. This strategy can therefore produce a “turn-on” type of fluorescent biosensor for GABA_AR ligands on the cell surface. Given that there are multiple distinct binding sites on the GABA_ARs, with binding to each site being dependent on the ligand structure (**Fig. 1**), it should be necessary to develop a suitable labeling reagent/quencher pair for each ligand site (**Fig. 2b, c**).

To covalently attach a fluorophore to GABA_ARs, I applied ligand-directed acyl imidazole (LDAI) chemistry^{27,28}. LDAI-based chemical labeling is driven by selective ligand-protein recognition, which facilitates acyl substitution reaction of the acyl imidazole group to nucleophilic amino acid residues (Lys, Ser, or Tyr) located near the ligand-binding domain (**Fig. 2a**). Importantly, the receptor function can be recovered after washing out the excess labeling reagents and the cleaved ligand moieties. In this study, I designed two LDAI reagents for labeling the different ligand-binding sites of the GABA_ARs, and subsequently defined them as chemical GABA_AR modification reagents (**CGAM-Gaba(1)** for the GABA site, **CGAM-Bzp(2)** for the benzodiazepine site) (**Fig. 2b**). **CGAM-Gaba** contained a gabazine derivative with orthosteric antagonistic activity ($IC_{50} = 11 \text{ nM}$)²⁹ as its ligand moiety. Flurazepam ($K_i = 170 \text{ nM}$)³⁰, which is a benzodiazepine derivative capable of acting as a partial positive allosteric modulator, was tethered as the ligand part of **CGAM-Bzp**. Using these labeling reagents (**CGAM-Gaba** and **CGAM-Bzp**), I covalently tether a fluorophore, Oregon Green (OG), to the GABA_ARs in close

proximity to the corresponding ligand-binding sites (i.e., the orthosteric GABA site, and the benzodiazepine site, respectively).

For the quencher-ligand conjugates, I employed QSY7³¹, which has been shown to exhibit a high quenching efficiency for OG over a broad range of emission wavelengths from 500 to 600 nm through a FRET mechanism. With the aim of achieving selective binding to targeted sites on the GABA_ARs, QSY7 was covalently attached to a ligand for a GABA or benzodiazepine site, affording **Gaba-Q(3)**, **Bzp-Q(4)**, **Bzp-M-Q(5)**, or **Bzp-L-Q(6)**, respectively (Fig. 2c).

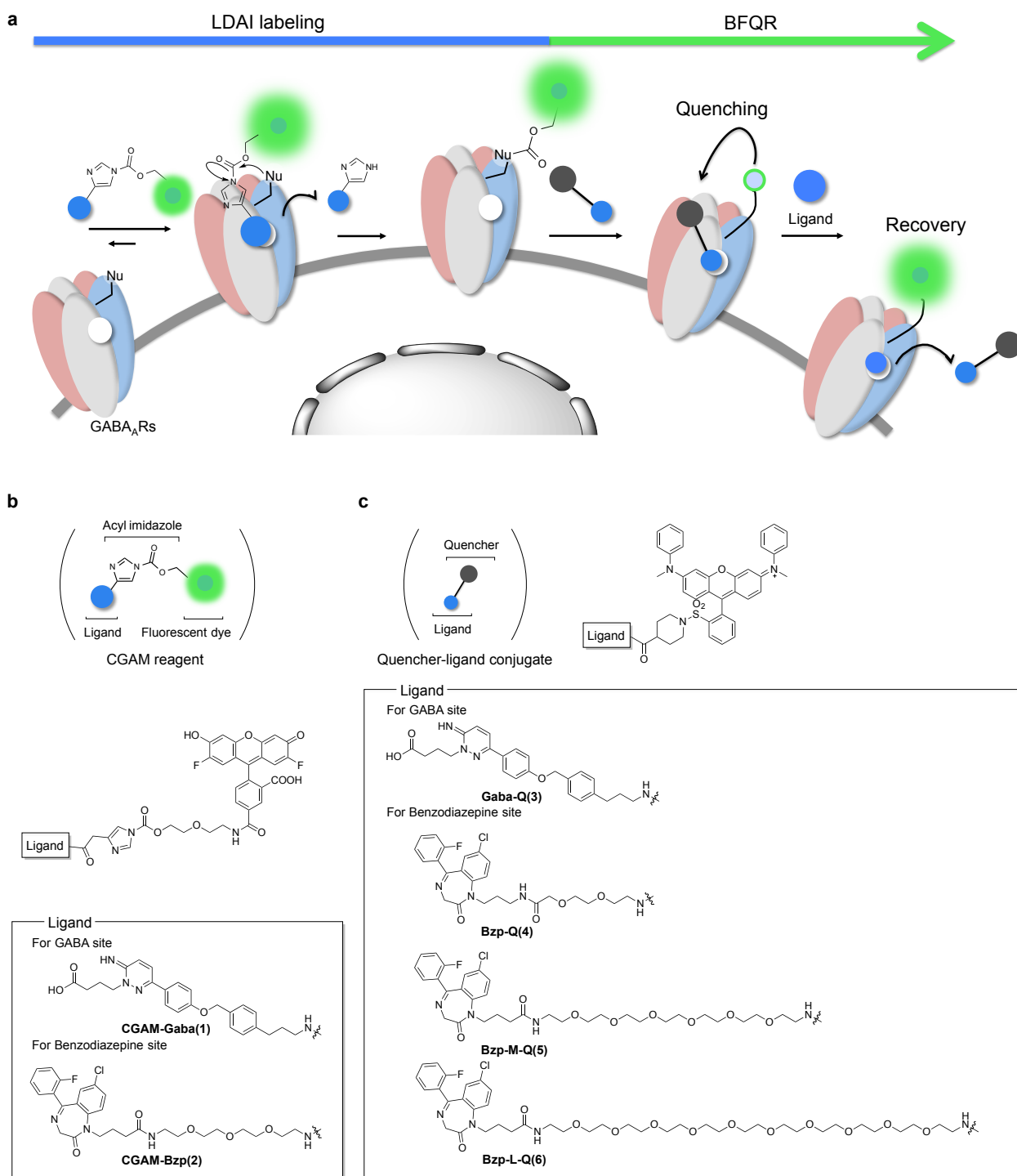


Fig. 2 Construction of GABA_AR-based semisynthetic fluorescent biosensors by coupling affinity based chemical labeling with BFQR system. (a) Schematic illustration for the construction of GABA_AR-based semisynthetic biosensors. Nu: nucleophile. (b) Chemical structures of **CGAM** reagents used in the present study. **CGAM-Gaba(1)**, and **CGAM-Bzp(2)** were used as labeling reagents for GABA-, and benzodiazepine-site, respectively. (c) Chemical structures of quencher-ligand conjugates used in the present study. **Gaba-Q(3)** and **Bzp-Q(4)**, **Bzp-M-Q(5)**, **Bzp-L-Q(6)** were used as a fluorescent quencher for GABA- and benzodiazepine-site, respectively.

1-2-2. Chemical labeling of GABA_ARs on live HEK293T cells using CGAM reagents

The recombinant expression systems in living cells that allow for the expression of GABA_AR subunits with controlled composition were used to develop my experiments. The reactions for the chemical labeling of the GABA_ARs were conducted on live HEK293T cells transfected with the $\alpha 1$, $\beta 3$, and $\gamma 2$ subunits of the GABA_ARs. First, I confirmed the chemical labeling of the GABA_ARs using **CGAM-Gaba**. After simple addition of **CGAM-Gaba** to culture medium and the subsequent incubation, the labeling reaction was analyzed by western blotting (WB) of the cell lysates using an anti-fluorescein/Oregon Green (anti-FI/OG) antibody. As shown in **Figure 3a**, WB revealed a band around 50 kDa in the $\alpha 1/\beta 3/\gamma 2$ GABA_AR-expressing cells which corresponded to the molecular weight of GABA_AR, and the labeled band could be assigned to the $\alpha 1$ or $\gamma 2$ subunit. Notably, this band was not observed in the cells transfected with a control vector lacking the genes associated with the GABA_ARs. Furthermore, this band did not appear in the presence of a competitive inhibitor (gabazine), which clearly indicated that the labeling was facilitated by an affinity-driven proximity effect. I further examined the subunit specificity for the labeling of the GABA_ARs using HEK293T cells transfected with varied compositions of the subunits (a single subunit of $\alpha 1$, $\beta 3$, $\gamma 2$; two subunits of $\alpha 1/\beta 3$, $\alpha 1/\gamma 2$, $\beta 3/\gamma 2$; and three subunits of $\alpha 1/\beta 3/\gamma 2$). Among them, the pentamers of β , α/β , α/γ , or β/γ were experimentally revealed to retain both their ion channel activity and ligand-binding capability^{32,33}. The labeled band around 50 kDa did not correspond to the $\beta 3$ subunit (55 kDa) and was only observed in the cells expressing $\alpha 1/\beta 3$ or $\beta 3/\gamma 2$ (**Fig. 3b**). This result was similar to that of the cells expressing $\alpha 1/\beta 3/\gamma 2$, and suggested that the $\alpha 1$ and $\gamma 2$ subunits had both been covalently labeled using **CGAM-Gaba**. Previous studies using photoaffinity labeling, point mutagenesis, and molecular modeling have revealed that the GABA-site is located at the extracellular α - β interface⁵. However, the present results suggest that the gabazine derivative, the ligand moiety of **CGAM-Gaba**, may bind to β - γ interface, as well as α - β interface. I also investigated the live imaging of **CGAM-Gaba**-treated cells by confocal microscopy. As shown in **Figure 3c**, labeled fluorescence was predominantly observed on the plasma membranes of $\alpha 1/\beta 3/\gamma 2$

GABA_AR-transfected cells. In good agreement with the WB analyses, the fluorescence was scarcely observed in the presence of competitive inhibitors or in the vector-transfected cells. Based on these results, I concluded that the GABA_ARs on the surfaces of living cells can be selectively labeled with OG using **CGAM-Gaba**.

I also investigated the fluorescent labeling of the benzodiazepine site of the GABA_ARs using **CGAM-Bzp**. WB analyses and confocal imaging results clearly showed that the selective labeling of GABA_ARs occurred by **CGAM-Bzp** in HEK293T cells transfected with $\alpha 1/\beta 3/\gamma 2$ GABA_ARs but not in the vector-transfected cells (**Fig. 3a, 3d**). I also confirmed that the labeling was completely inhibited by the co-administration of a competitive inhibitor (flumazenil) even in the GABA_AR-transfected cells. In contrast to the **CGAM-Gaba** labeling of the GABA site, the chemical labeling by **CGAM-Bzp** required all three components (i.e., $\alpha 1$, $\beta 3$, and $\gamma 2$) of the GABA_ARs (**Fig. 3b**). The subsequent peptide mass-fingerprinting analyses revealed that Lys184 on loop F in the $\gamma 2$ -subunit was modified with OG in $\alpha 1/\beta 3/\gamma 2$ GABA_AR-transfected cells (**Fig. 3e-g**). According to the previous molecular model study³⁴, the labeled Lys residue locates near the proposed benzodiazepine-binding site, indicating that chemical labeling using **CGAM-Bzp** is indeed driven by the proximity effect with the ligand-protein recognition.

Taken together, these results clearly show that vicinity of the ligand binding sites of GABA_ARs can be covalently labeled with a fluorophore using **CGAM** reagents bearing an appropriate ligand for the orthosteric or allosteric sites on living cells. Most notably, this process can be achieved without the assistance of any structural or sequence information.

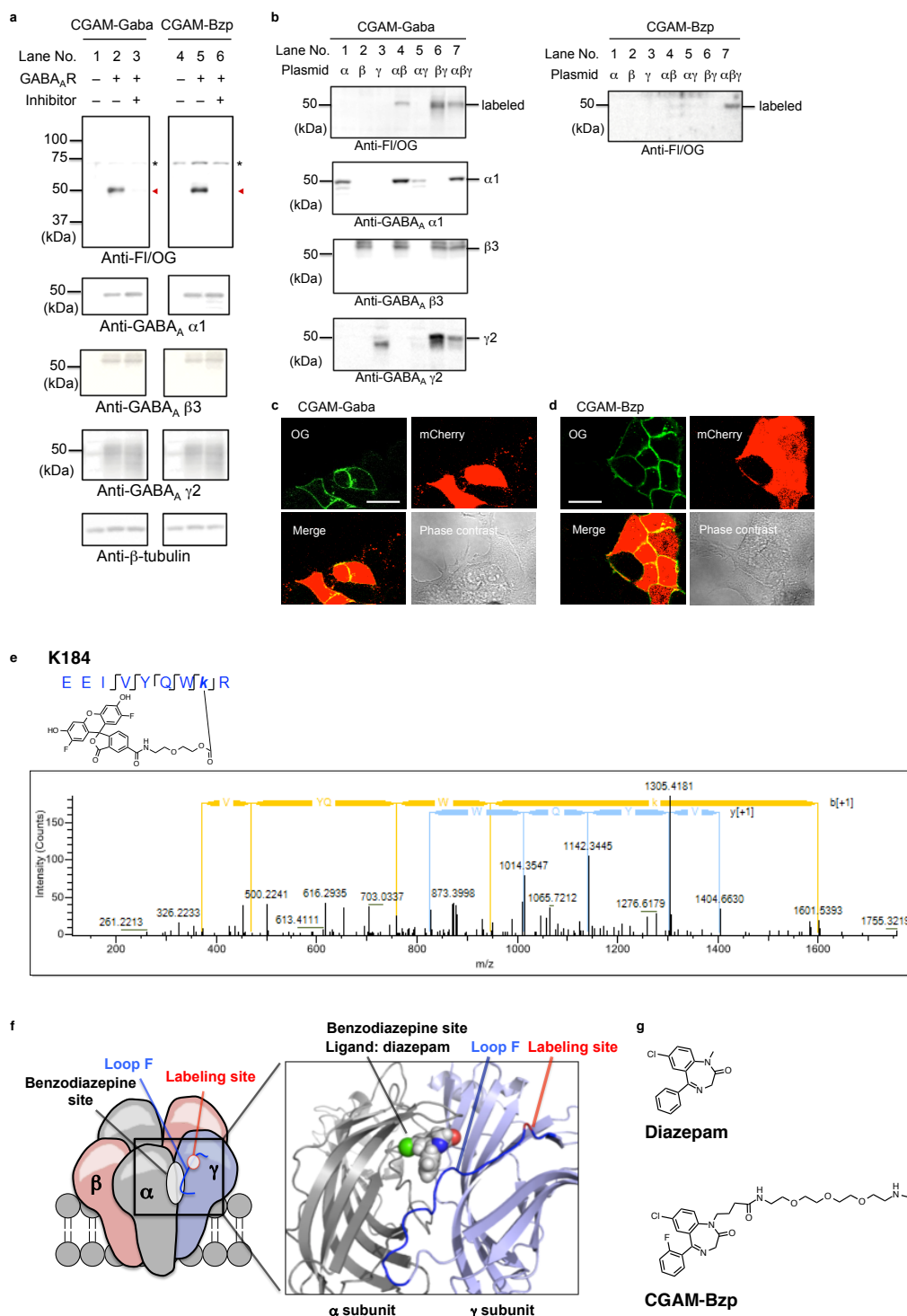


Fig. 3 Chemical labeling of GABA_ARs in HEK293T cells using CGAM reagents. (a) WB analyses of HEK293T cells labeled by **CGAM** reagents. For GABA-site labeling, HEK293T cells transfected with $\alpha 1/\beta 3/\gamma 2$ GABA_ARs (GABA_AR(+)) or the control vector (GABA_AR(-)) were treated with 1 μ M **CGAM-Gaba** in the presence or absence of 20 μ M gabazine. For benzodiazepine site labeling, the cells were treated with 1 μ M **CGAM-Bzp** in the presence or absence of 20 μ M flumazenil. These reaction are conducted in serum free DMEM at 37 °C for 3 h. Red arrowhead indicates labeled GABA_ARs. * indicates non-specific labeling to bovine serum albumin (BSA) included in the culture medium. **(b)** Subunit specificity for GABA_AR labeling

using **CGAM-Gaba** or **CGAM-Bzp**. HEK293T cells transfected with varied compositions of the GABA_AR subunits (a single subunit of $\alpha 1$, $\beta 3$, $\gamma 2$, two subunits of $\alpha 1/\beta 3$, $\alpha 1/\gamma 2$, $\beta 3/\gamma 2$ or three subunits of $\alpha 1/\beta 3/\gamma 2$) were treated with 0.5 μ M **CGAM-Gaba** or 0.5 μ M **CGAM-Bzp** in serum free DMEM at 37 °C for 4 h. (**c**, **d**) Confocal live imaging of HEK293T cells labeled with **CGAM-Gaba** in **c** or **CGAM-Bzp** in **d**. Labeling was conducted with the same procedure as described in **a**. mCherry-N1 was utilized as a transfection marker. Scale bars, 20 μ m. (**e**) MS/MS analysis of the OG-modified peptide detected from $\gamma 2$ subunit of OG-labeled GABA_ARs. (**f**) Structural illustration and structure model based on homology models and docking studies of GABA_AR (nchembio.917-s2.pdb). Loop F and the amino acids modified by **CGAM-Bzp** are shown as blue and red cartoon, respectively. Hydrogen, nitrogen, oxygen, and chlorine atom of diazepam ligand are shown as gray, blue, red, and green sphere, respectively. (**g**) Comparison of the chemical structure of diazepam and **CGAM-Bzp**.

1-2-3. Construction of GABA_AR-based semisynthetic fluorescent biosensors for orthosteric ligands

Using the OG-labeled GABA_ARs, I proceeded to construct a series of semisynthetic biosensors capable of responding to small molecules bound to each ligand-binding site with a “turn-on” fluorescence mode, according to the BFQR scheme. To sense orthosteric GABA-site ligands of the GABA_ARs, I used a pair composed of **CGAM-Gaba** as the labeling reagent and **Gaba-Q** as the corresponding quencher (**Fig. 4a**). After labeling the $\alpha 1/\beta 3/\gamma 2$ GABA_ARs with **CGAM-Gaba**, the culture medium was washed several times. Upon the addition of **Gaba-Q**, the fluorescence of the plasma membrane of the HEK293T cells gradually decreased in a concentration-dependent manner ($K_d = 33$ nM) (**Fig. 4a-c**). The fluorescence intensity could then be recovered by the addition of various types of GABA_AR orthosteric ligands in the presence of **Gaba-Q** (**Fig. 4a,b,e**). As shown in **Figure 4d and 4e**, substantial fluorescence recovery was observed in the presence of GABA, which is an orthosteric GABA-site agonist. Gabazine, which is an orthosteric antagonist, also produced a similar effect. In contrast, flumazenil, amobarbital, picrotoxin, allopregnanolone, and etomidate, which are allosteric modulators of the GABA_ARs, induced only negligible changes in the fluorescence. These results therefore indicated that my fluorescent biosensor can specifically discriminate GABA-site ligands from those designed to interact with other sites. Furthermore, the dissociation constants of these ligands (K_d) could be determined using the apparent affinity constant (K_{app}) directly obtained from the titration curve, and the K_d value of **Gaba-Q** (for details, see Experimental Section) (**Fig. 4f**). The resulting K_d values for GABA and gabazine were 210 μ M and 830 nM, respectively. These values were larger but still comparable to those reported previously ($EC_{50} = 26\text{--}41$ μ M for GABA^{30,35} and $IC_{50} = 300\text{--}350$ nM for gabazine^{24,29}).

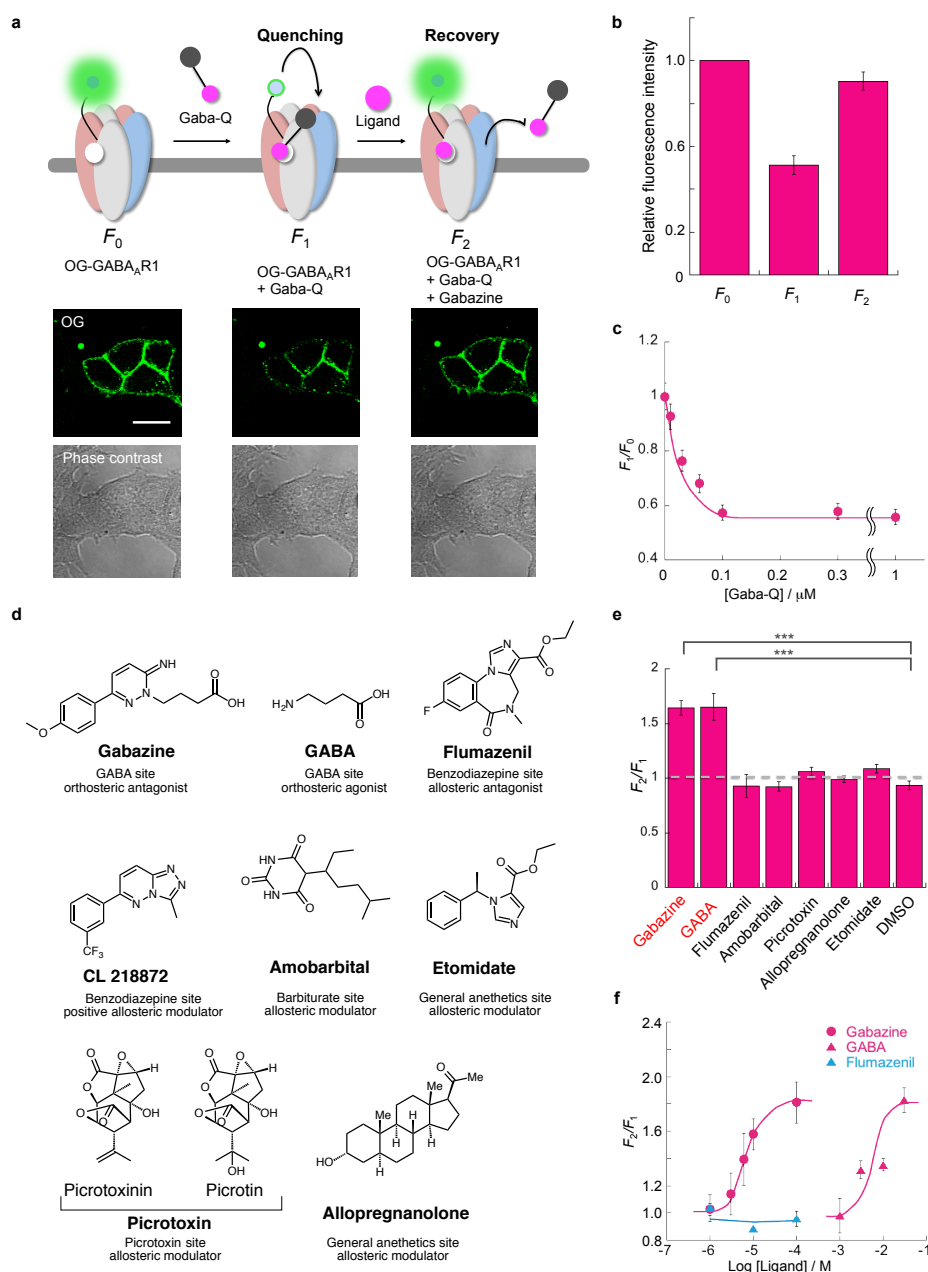


Fig. 4 Construction of GABA_AR-based semisynthetic fluorescent biosensors for orthosteric ligands. (a) Fluorescence quenching of OG-labeled GABA_AR by **Gaba-Q** and the recovery by orthosteric ligands. Schematic illustration of the fluorescent changes (upper) and representative confocal images (lower) are shown. HEK293T cells transfected with $\alpha 1/\beta 3/\gamma 2$ GABA_ARs were labeled by 2 μ M **CGAM-Gaba** to obtain OG-labeled GABA_ARs (OG-GABA_AR1). The cells were treated with 0.1 μ M **Gaba-Q**, which was followed by the addition of 200 μ M gabazine. Scale bars, 20 μ m. (b) Normalized labeled fluorescence of OG-GABA_AR1 (F_0), the quenched fluorescence by **Gaba-Q** (F_1), and the recovered fluorescence after addition of gabazine (F_2). The fluorescent intensity was obtained from confocal imaging as described in **a**. $n = 6$. (c) Concentration-dependency of **Gaba-Q** for the fluorescent quenching ratio (F_1/F_0) of

OG-GABA_AR1. The K_d value (33 nM) was directly calculated from the curve. $n = 3$. **(d)** Chemical structure of representative orthosteric or allosteric modulators for GABA_ARs utilized in this study. **(e)** Specificity of the fluorescent biosensor for various types of GABA_AR ligands. The fluorescent recovery ratio (F_2/F_1) after addition of each ligand is shown. Each ligand concentration is the following: gabazine (1 mM), GABA (10 mM), flumazenil (0.2 mM), amobarbital (1 mM), picrotoxin (1 mM), allopregnanolone (0.05 mM), and etomidate (1 mM). $n = 6$. **(f)** Concentration-dependency of orthosteric agonist and antagonist for the F_2/F_1 value of OG-GABA_AR1. The labeled cells were treated with 0.1 μ M **Gaba-Q**, which was followed by the addition of various concentration of gabazine, GABA, or flumazenil. $n = 3$. *** $P < 0.001$. Data represent mean $\pm$ SEM.

1-2-4. Construction of GABA_AR-based semisynthetic fluorescent biosensors for allosteric ligands

This method was also applied to construct fluorescent biosensors for benzodiazepine-site ligands using **CGAM-Bzp** and **Bzp-Q** in HEK 293T cells (**Fig. 5a**). The addition of **Bzp-Q** to the OG-tethered GABA_ARs prepared with **CGAM-Bzp** resulted in a concentration-dependent decrease in their fluorescence ($K_d = 50$ nM) (**Fig. 5a-c**). The subsequent addition of flumazenil, which is a well-known benzodiazepine-site ligand, led to a recovery in the fluorescence (**Fig. 4d and Fig. 5a,b,d**). Interestingly, the addition of CL218872, whose structure is obviously different to that of flumazenil, also led to the recovery of the fluorescence. In contrast, the addition of GABA-site ligands (e.g., gabazine) or ligands capable of binding to other allosteric sites (e.g., amobarbital, picrotoxin, allopregnanolone, and etomidate) did not show significant changes on the fluorescence. As was the case with the GABA-site ligands, careful titration experiments provided the dissociation constants for flumazenil ($K_d = 7.7$ nM) and CL218872 ($K_d = 2.1$ μ M) from the concentration-dependent titration curves (**Fig. 5e**). Notably, these values were in a similar range to those reported previously ($K_i = 0.6$ – 5 nM and 0.4 μ M for flumazenil^{30,36} and CL218872³⁷, respectively). Although CL218872 is normally categorized as a non-benzodiazepine ligand³⁸, it has been reported to bind to the benzodiazepine site through a competitive binding assay using radioisotope-labeled benzodiazepine derivatives, which is consistent with my results.

Next, I evaluated quenching efficacy of three quencher-ligand conjugates for benzodiazepine site bearing the different spacer lengths (**Bzp-Q**, **Bzp-M-Q**, **Bzp-L-Q**) using OG-labeled GABA_ARs. As shown in **Fig. 2c and 5f**, the quenching efficiency is dependent on the spacer, that is, the shortest one (**Bzp-Q**) was the most efficient. Thus, the spacer length of quencher-ligand conjugates is one of the key factors for controlling quenching efficacy.

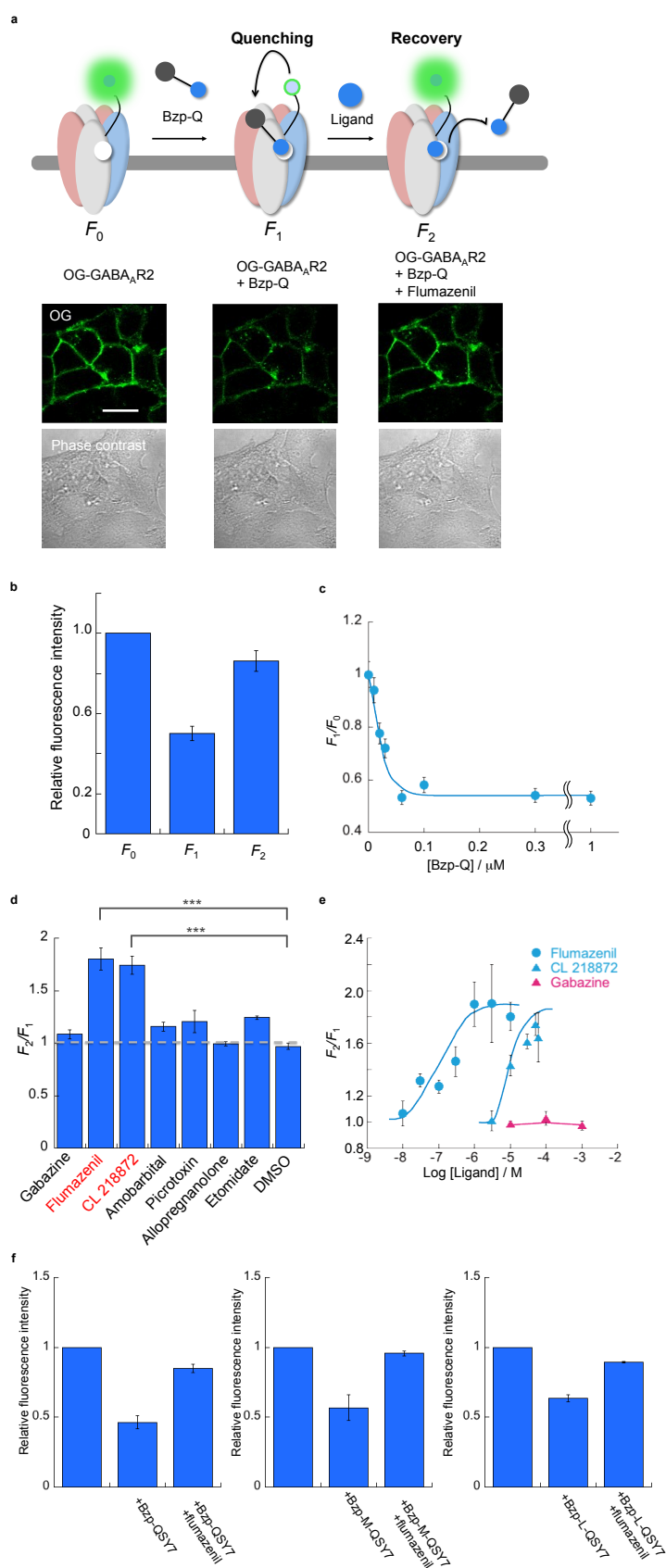


Fig. 5 Construction of GABA_AR-based fluorescent biosensors for allosteric benzodiazepine site ligands. (a) Fluorescence quenching of OG-labeled GABA_AR by 1 μ M Bzp-Q and the

recovery by 200 μ M flumazenil. Schematic illustration (upper) and representative confocal images (lower) are shown. HEK293T cells transfected with $\alpha 1/\beta 3/\gamma 2$ GABA_ARs were labeled by 2 μ M **CGAM-Bzp** (OG-GABA_AR2). Scale bars, 20 μ m. **(b)** Relative intensity of labeled (F_0), quenched (F_1), and recovered fluorescence (F_2). The fluorescent intensity was obtained from confocal imaging. $n = 3$. **(c)** Concentration-dependency of **Bzp-Q** for the F_1/F_0 ratio of OG-GABA_AR2. The K_d value (50 nM) was directly calculated from the curve. $n = 3$. **(d)** Specificity of the fluorescent biosensor for GABA_AR ligands. The F_2/F_1 ratio after addition of each ligand is shown. Each ligand concentration is the following: gabazine (1 mM), flumazenil (10 μ M), CL217782 (0.05 mM), amobarbital (1 mM), picrotoxin (1 mM), allopregnanolone (0.05 mM), and etomidate (1 mM). $n = 3$ **(e)** Concentration-dependency of GABA_AR ligands for the F_2/F_1 value of OG-GABA_AR2. The labeled cells were treated with 1 μ M **Bzp-Q**, which was followed by the addition of various concentration of flumazenil, CL218872, or gabazine. $n = 3$. **(f)** Effect of the spacer length of the quencher-ligand conjugates for benzodiazepine site for the relative fluorescence intensity of OG-GABA_AR2. The labeled cells were treated with 1 μ M the quencher-ligand conjugates for benzodiazepine site (**Bzp-Q**, **Bzp-M-Q**, **Bzp-L-Q**), which was followed by the addition of 10 μ M flumazenil. *** $P < 0.001$. Data represent mean $\pm$ SEM.

1-2-5. Discriminating subtype-specific ligands using GABA_AR-based fluorescent biosensors.

Moreover, I expected that the present method should have a potential to construct fluorescent biosensors capable of discriminating subtype-selective drugs. To demonstrate this possibility, I changed the subtype composition of GABA_ARs and labeled them as follows. HEK293T cells were transfected with expression vectors for $\alpha 1/\beta 3/\gamma 2$, $\alpha 3/\beta 3/\gamma 2$, or $\alpha 5/\beta 3/\gamma 2$ to produce $\alpha 1$ -, $\alpha 3$ -, or $\alpha 5$ -containing GABA_ARs, respectively. According to the same protocol for $\alpha 1$ -containing GABA_ARs, $\alpha 3$ - or $\alpha 5$ -containing GABA_ARs expressed on HEK293T cells were successfully converted into fluorescent biosensors using **CGAM-Bzp** and **Bzp-Q** (Figs. 6a,b). As shown in **Figure 6c**, the F_2/F_1 ratio was selectively increased by addition of $\alpha 1$ -selective Zolpidem³⁸ or $\alpha 5$ -selective L655708³⁹ in this sensor system expressing $\alpha 1$ - or $\alpha 5$ -containing GABA_ARs, respectively. On the other hand, flumazenil, a subtype non-selective benzodiazepine derivative³⁸ caused the increases in the F_2/F_1 ratio for all of the three sensor systems. This proof-of-principle experiments clearly revealed that the present strategy can be applied to screening of the subtype-selective small molecules for GABA_ARs

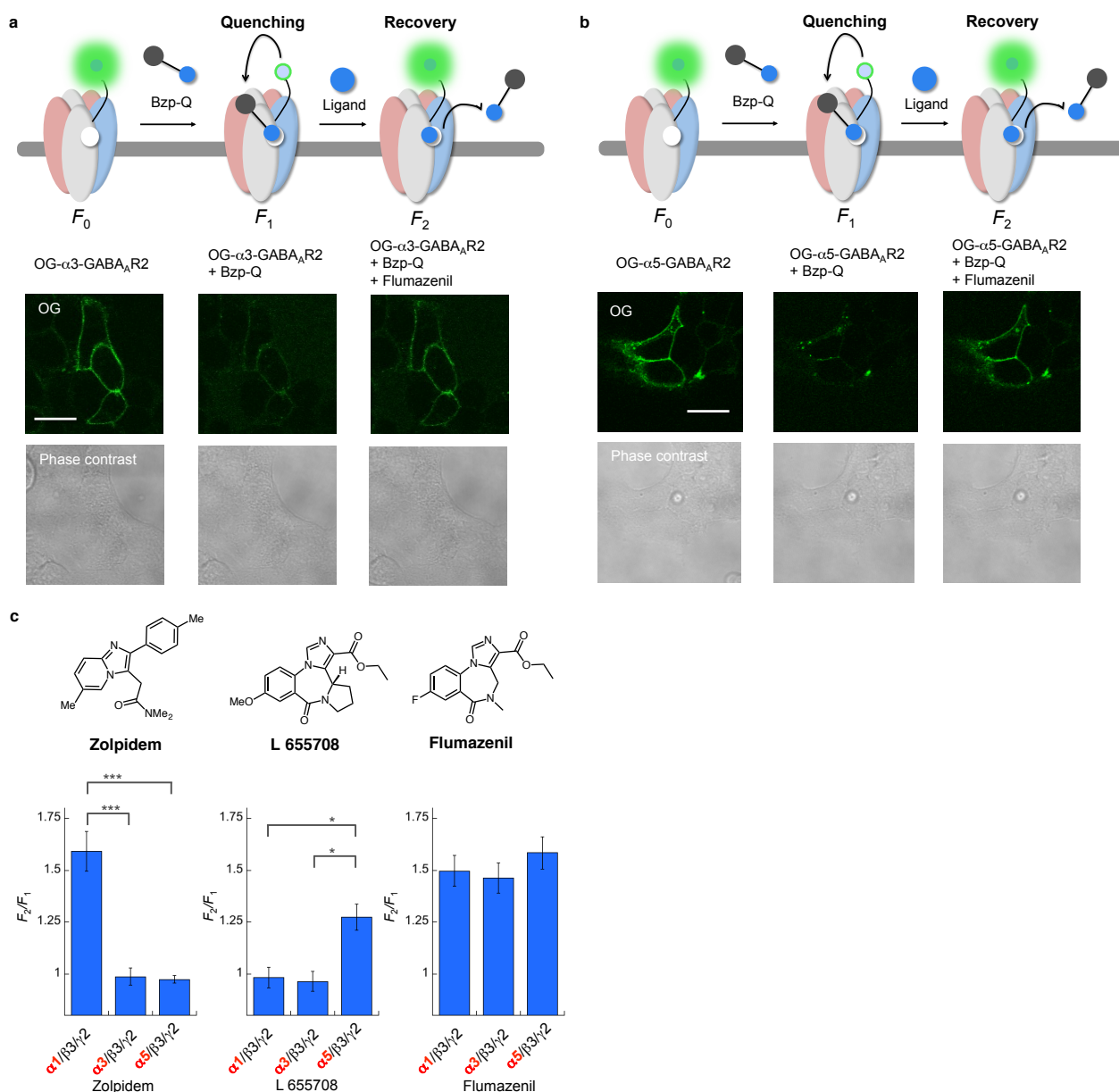


Fig. 6 Construction of $\alpha 3$ or $\alpha 5/\beta 3/\gamma 2$ GABA_AR-based semisynthetic fluorescent biosensors for benzodiazepine site ligands. (a) Fluorescence quenching of OG-labeled $\alpha 3/\beta 3/\gamma 2$ GABA_AR by **Bzp-Q** and the recovery by flumazenil. Schematic illustration of the fluorescent changes (upper) and representative confocal images (lower) are shown. HEK293T cells transfected with $\alpha 3/\beta 3/\gamma 2$ GABA_ARs were labeled by 2 μ M **CGAM-Bzp** to obtain OG-labeled $\alpha 3/\beta 3/\gamma 2$ GABA_ARs (OG- $\alpha 3$ -GABA_AR2). The cells were treated with 1 μ M **Bzp-Q**, which was followed by the addition of 10 μ M flumazenil. (b) Fluorescence quenching of OG-labeled $\alpha 5/\beta 3/\gamma 2$ GABA_AR by **Bzp-Q** and the recovery by flumazenil. Schematic illustration of the fluorescent changes (upper) and representative confocal images (lower) are shown. HEK293T cells transfected with $\alpha 5/\beta 3/\gamma 2$ GABA_ARs were labeled by 2 μ M **CGAM-Bzp** to obtain OG-labeled $\alpha 5/\beta 3/\gamma 2$ GABA_ARs (OG- $\alpha 5$ -GABA_AR2). The cells were treated with 1 μ M **Bzp-Q**, which was followed by the addition of 10 μ M flumazenil. Scale bars, 20 μ m. (c) Subtype-selectivity of the $\alpha 1$ -

$\alpha 3$ -, or $\alpha 5$ -containing fluorescent biosensor for benzodiazepine site ligands. The F_2/F_1 ratio after addition of each ligand is shown. Each ligand concentration is the following: zolpidem (5 μ M), L655708 (0.1 μ M), and flumazenil (1 μ M). $n = 3$. * $P < 0.05$; *** $P < 0.001$. Data represent mean $\pm$ SEM.

1-3. Conclusion.

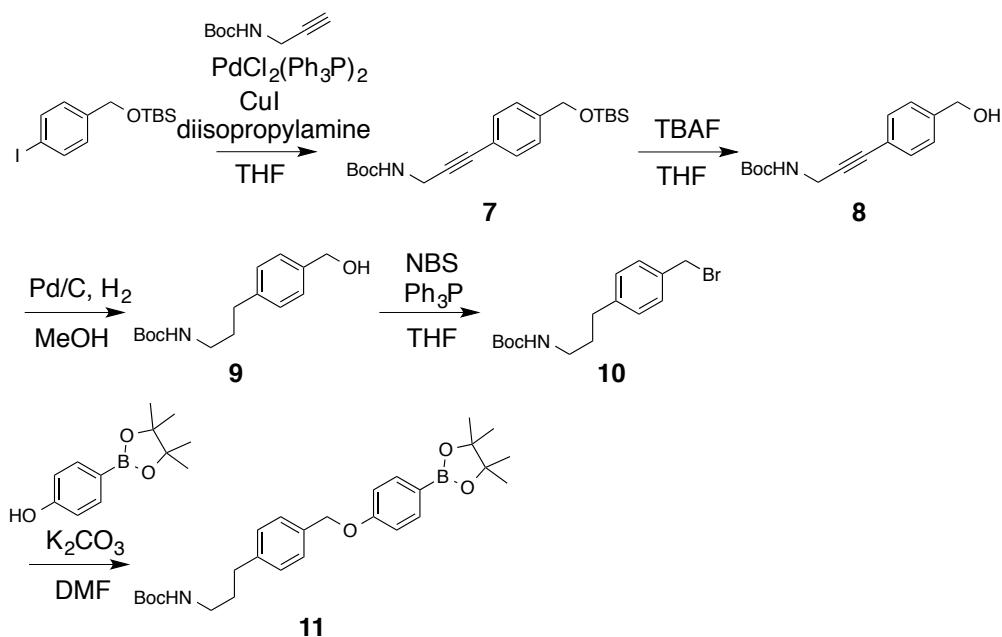
I have successfully constructed fluorescent biosensors for GABA_AR ligands by coupling ligand-directed chemical labeling with a BFQR-based fluorescence turn-on mechanism. The appropriate combination of the **CGAM** reagents with the quencher-ligand conjugates led to the successful formation of “turn-on” type fluorescent biosensors for the orthosteric and allosteric GABA_AR ligands on living cells. In contrast to the other methods available for the semisynthesis of fluorescent biosensors, which are strongly reliant on the availability of structural data pertaining to the target proteins, my strategy only requires an appropriate ligand. Using this method, complicated (membrane-bound) proteins with multiple binding sites, such as GABA_ARs, can be converted to fluorescent turn-on biosensors for ligands that bind efficiently to the target protein.

There are significant differences in the expression levels of the GABA_AR subtypes in the different regions of the brain, which are associated with distinct physiological effects^{4,40}. The subtype-selective ligands could therefore be useful as therapeutic agents for the treatment of anxiety, sleep disorders, convulsions, and memory deficits with very few side effects. For example, a benzodiazepine derivative, TPA023, which acts as an $\alpha 2/\alpha 3$ -subtype selective positive allosteric modulator, has been demonstrated to have anxiolytic but not sedative effects⁴¹. L655708, an $\alpha 5$ -subtype selective benzodiazepine derivative having partial inverse agonistic efficacy, enhanced cognition without affecting proconvulsant and anxiogenic activities⁴². In this study, I clearly revealed that my method can flexibly construct biosensors composed of $\alpha 1$ -, $\alpha 3$ -, or $\alpha 5$ -containing GABA_ARs for benzodiazepine-site ligands, and $\alpha 1$ - or $\alpha 5$ -subtype selective drug was indeed detected using these sensors. Therefore, this ligand screening system can be extended as a versatile platform to the screening of subtype-specific ligands to selectively treat these neurological disorders.

1-4. Experimental Section.

General materials and methods for organic synthesis

All chemical reagents and solvents were purchased from commercial sources (Wako pure chemical, TCI chemical, Sigma-Aldrich, Sasaki Chemical) and were used without further purification. Thin layer chromatography (TLC) was performed on silica gel 60 F254 precoated aluminum sheets (Merck). Chromatographic purification was performed using flash column chromatography on silica gel 60 N (neutral, 40–50 μm , Kanto Chemical). ^1H -NMR spectra were recorded on 400 MHz Varian Mercury. ^{13}C -NMR spectra were recorded on 600 MHz JNM-ECA. Chemical shifts were referenced to residual solvent peaks or tetramethylsilane ($\delta = 0$ ppm). High resolution mass spectra were measured on an Exactive (Thermo Scientific) equipped with electron spray ionization (ESI). Reversed-phase HPLC (RP-HPLC) was carried out on a Hitachi Chromaster system equipped with a diode array, and an YMC-Pack Triat C18 or ODS-A column.

Synthesis of **11**.***Tert*-butyl (3-(4-(((*tert*-butyldimethylsilyl)oxy)methyl)phenyl)prop-2-yn-1-yl)carbamate (**7**)**

To a stirred solution of *tert*-butyl((4-iodobenzyl)oxy)dimethylsilane (1.49 g, 4.27 mmol) in THF (43 ml) was added *tert*-butyl prop-2-yn-1-ylcarbamate (795 mg, 5.13 mmol), diisopropylamine (1.8 ml, 12.8 mmol). Then, $\text{PdCl}_2(\text{PPh}_3)_2$ (596 mg, 0.85 mmol), CuI (323 mg, 1.7 mmol) were added to the solution under argon atmosphere. The reaction mixture was allowed to stir for 1 h at room temperature. The solution was diluted with CHCl_3 and washed with 1N HCl. The organic layer was dried over Na_2SO_4 , filtered, evaporated. The residue was purified by silica gel column chromatography (Hexane only $\rightarrow$ Hexane : AcOEt = 20:1) to yield compound **7** (1.4 g, 3.73 mmol, 87 %) as a clear oil. $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 7.37 (d, 2H, $J = 6.0$ Hz), 7.25 (d, 2H, $J = 6.0$ Hz), 4.73 (s, 2H), 4.15 (brs, 2H), 1.47 (s, 9H), 0.94 (s, 9H), 0.09 (s, 6H).

***Tert*-butyl (3-(4-(hydroxymethyl)phenyl)prop-2-yn-1-yl)carbamate (**8**)**

To a stirred solution of compound **7** (1.4 g, 3.73 mmol) in THF (37 ml) was added TBAF (1.95 g, 7.46 mmol). The reaction mixture was allowed to stir for 2 h at room temperature. The solution was diluted with CHCl_3 and washed with H_2O . The organic layer was dried over Na_2SO_4 , filtered,

evaporated. The residue was purified by silica gel column chromatography (Hexane only → Hexane : AcOEt = 2:1) to yield compound **8** (950 mg, 3.63 mmol, 96 %) as a clear oil. ¹H-NMR (400 MHz, CDCl₃): δ 7.33 (d, 2H, *J* = 8.0 Hz), 7.23 (d, 2H, *J* = 8.0 Hz), 5.07 (brs, 1H), 4.59 (s, 2H), 4.07 (brs, 2H), 3.31 (brs, 1H), 1.43 (s, 9H).

***Tert*-butyl (3-(4-(bromomethyl)phenyl)propyl)carbamate (10)**

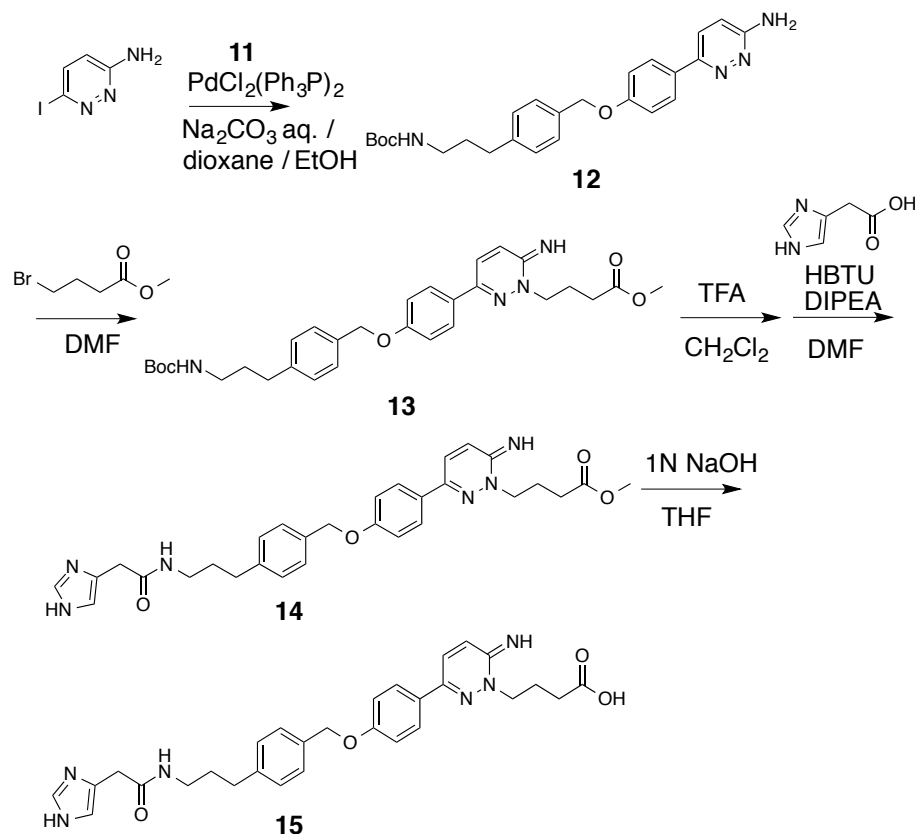
To a stirred solution of compound **8** (950 mg, 3.58 mmol) in MeOH (36 ml) was added 10 % *palladium-carbon* (100 mg) at room temperature. The reaction mixture was allowed to stir for 16 h under hydrogen atmosphere. The solution was filtered by Celite and evaporated to give compound **9**. The compound **9** was dissolved in dry THF (36 ml). Then, Ph₃P (2.96 g, 11.3 mmol), *N*-Bromosuccinimide (2 g, 11.3 mmol) were added to the solution at 0 °C. The reaction mixture was allowed to stir for 20 min at room temperature. Then, The reaction mixture was diluted with CHCl₃ and washed with H₂O. The organic layer was dried over Na₂SO₄, filtered, evaporated. The residue was purified by silica gel column chromatography (Hexane only → Hexane : CHCl₃ = 3:1 → Hexane : AcOEt = 10:1) to yield compound **10** (656 mg, 2.0 mmol, 53 %) as a white solid. ¹H-NMR (400 MHz, CDCl₃): δ 7.05-7.31 (m, 4H), 4.48 (s, 2H), 3.13 (m, 2H), 2.61 (m, 2H), 1.79 (m, 2H), 1.44 (s, 9H).

***Tert*-butyl (3-(4-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy)methyl)phenyl)propyl)carbamate (11)**

To a stirred solution of K₂CO₃ (380 mg, 2.74 mmol) in dry DMF (4.6 ml) was added 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenol (200 mg, 0.91 mmol), compound **10** (300 mg, 0.91 mmol). The reaction mixture was allowed to stir for 6 h at 80 °C. The solution was diluted with diethyl ether and washed with H₂O. The organic layer was dried over Na₂SO₄, filtered, evaporated. The residue was purified by silica gel column chromatography (Hexane only → Hexane : AcOEt = 20:1 → Hexane : AcOEt = 10:1) to yield compound **11** (170 mg, 0.36 mmol,

40 %) as a clear oil. $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 7.74 (d, 2H, $J = 8.0$ Hz), 7.33 (d, 2H, $J = 8.0$ Hz), 7.18 (d, 2H, $J = 8.0$ Hz), 6.95 (d, 2H, $J = 7.6$ Hz), 5.04 (s, 2H), 4.52 (brs, 1H), 3.14 (m, 2H), 2.64 (t, 2H, $J = 8.0$ Hz), 1.80 (t, 2H, $J = 8.0$ Hz), 1.44 (s, 9H), 1.32 (s, 12H).

Synthesis of **15**.



Tert-butyl (3-(4-((4-(6-aminopyridazin-3-yl)phenoxy)methyl)phenyl)propyl)carbamate (12**)**

To a stirred solution of 6-iodopyridazin-3-amine (70 mg, 0.32 mmol) in 2M Na_2CO_3 : dioxane : EtOH = 1 : 1 : 2 (6.4 ml) was added compound **11** (75 mg, 0.16 mmol), $\text{PdCl}_2(\text{Ph}_3\text{P})_2$ (23 mg, 32.0 μmol) under argon atmosphere. The reaction mixture was allowed to stir for 30 min at 100 $^\circ\text{C}$. The solution was diluted with CHCl_3 and washed with H_2O . The organic layer was dried over Na_2SO_4 , filtered, evaporated. The residue was purified by silica gel column chromatography (CHCl_3 only $\rightarrow \text{CHCl}_3$: MeOH = 20:1 $\rightarrow \text{CHCl}_3$: MeOH = 10:1) to yield compound **12** (19 mg, 44.0 μmol , 27 %) as a clear solid. $^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 7.75 (d, 2H, $J = 8.4$ Hz), 7.60

(d, 1H, $J = 9.2$ Hz), 7.33 (d, 2H, $J = 8.0$ Hz), 7.18 (d, 2H, $J = 8.0$ Hz), 7.03 (d, 2H, $J = 8.4$ Hz), 6.92 (d, 1H, $J = 9.2$ Hz), 5.05 (s, 2H), 3.07 (m, 2H), 2.61 (t, 2H, $J = 8.0$ Hz), 1.77 (t, 2H, $J = 7.6$ Hz), 1.40 (s, 9H).

Methyl

4-(3-(4-((4-(3-((*tert*-butoxycarbonyl)amino)propyl)benzyl)oxy)phenyl)-6-iminopyridazin-1(6H)-yl)butanoate (13)

To a stirred solution of compound **12** (19 mg, 43.0 μ mol) in dry DMF (874 μ l) was added methyl 4-bromobutanoate (78 mg, 0.43 mmol). The reaction mixture was allowed to stir for 2 h at 100 °C. The mixture was purified by silica gel column chromatography (CHCl_3 only $\rightarrow$ CHCl_3 : MeOH = 10:1 $\rightarrow$ MeOH only) to yield compound **13** (12 mg, 22.0 μ mol, 52 %) as a clear solid. ^1H -NMR (400 MHz, CD_3OD): δ 8.18 (d, 1H, $J = 9.6$ Hz), 7.85 (d, 2H, $J = 8.4$ Hz), 7.65 (d, 1H, $J = 9.6$ Hz), 7.33 (d, 2H, $J = 8.4$ Hz), 7.20 (d, 2H, $J = 8.0$ Hz), 7.09 (d, 2H, $J = 8.8$ Hz), 5.10 (s, 2H), 4.41 (t, 2H, $J = 6.8$ Hz), 3.60 (s, 3H), 3.06 (t, 2H, $J = 6.4$ Hz), 2.62 (t, 2H, $J = 6.4$ Hz), 2.56 (t, 2H, $J = 6.4$ Hz), 2.23 (t, 2H, $J = 6.8$ Hz), 1.77 (t, 2H, $J = 7.2$ Hz), 1.41 (s, 9H).

Methyl

4-(3-(4-((4-(3-(2-(1H-imidazol-4-yl)acetamido)propyl)benzyl)oxy)phenyl)-6-iminopyridazin-1(6H)-yl)butanoate (14)

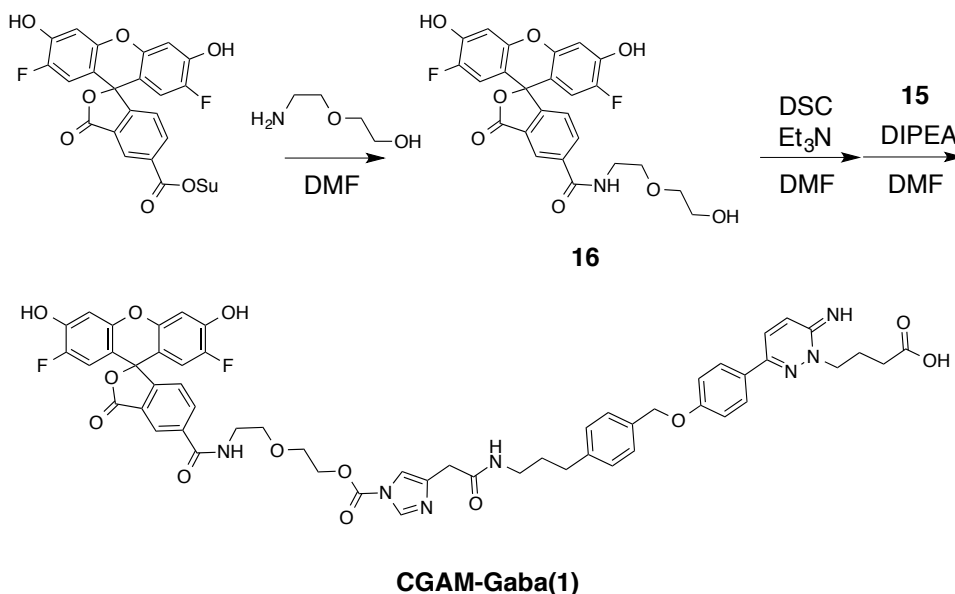
To a stirred solution of compound **13** (3 mg, 5.6 μ mol) in dry CH_2Cl_2 (1 ml) was added TFA (0.5 ml). The reaction mixture was allowed to stir for 30 min at room temperature. After azeotropic removal of TFA with toluene, the residue was dissolved in dry DMF (120 μ l). Then, 2-(1H-imidazol-4-yl)acetic acid (1.4 mg, 8.4 μ mol), HBTU (3.6 mg, 11 μ mol), DIPEA (10 μ l, 56 μ mol) were added to the solution. The reaction mixture was allowed to stir for 30 min at room temperature. The mixture was purified by HPLC (mobile phase; 0.1 % TFA CH_3CN (solvent A), 0.1 % TFA H_2O (solvent B)) to yield compound **14** (1.0 mg, 1.8 μ mol, 88 %) as a clear solid.

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 8.83 (s, 1H), 8.27 (d, 1H, $J = 10.0$ Hz), 7.94 (d, 2H, $J = 8.8$ Hz), 7.57 (d, 1H, $J = 9.2$ Hz), 7.40 (s, 1H), 7.38 (d, 2H, $J = 7.6$ Hz), 7.24 (d, 2H, $J = 8.0$ Hz), 7.15 (d, 2H, $J = 8.8$ Hz), 5.13 (s, 2H), 4.42 (t, 2H, $J = 7.2$ Hz), 3.71 (s, 2H), 3.57 (s, 3H), 3.24 (t, 2H, $J = 6.8$ Hz), 2.67 (t, 2H, $J = 7.6$ Hz), 2.58 (t, 2H, $J = 7.2$ Hz), 2.25 (t, 2H, $J = 6.8$ Hz), 1.85 (t, 2H, $J = 6.4$ Hz).

4-(3-(4-((4-(3-(2-(1H-imidazol-4-yl)acetamido)propyl)benzyl)oxy)phenyl)-6-iminopyridazin-1(6H)-yl)butanoic acid (15)

To a stirred solution of compound **14** (3 mg, 5.6 μmol) in THF (1 ml) and H_2O (800 μl) was added 1N NaOH (200 μl). The reaction mixture was allowed to stir for 3 h at 50 $^\circ\text{C}$. The solution was neutralized with 1N HCl (200 μL). The solvent was evaporated to yield compound **15** (quant.) as a white solid. The crude compound **15** was used directly for the next step without further purification.

Synthesis of CGAM-Gaba(1).



2',7'-difluoro-3',6'-dihydroxy-N-(2-(2-hydroxyethoxy)ethyl)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-5-carboxamide (16)

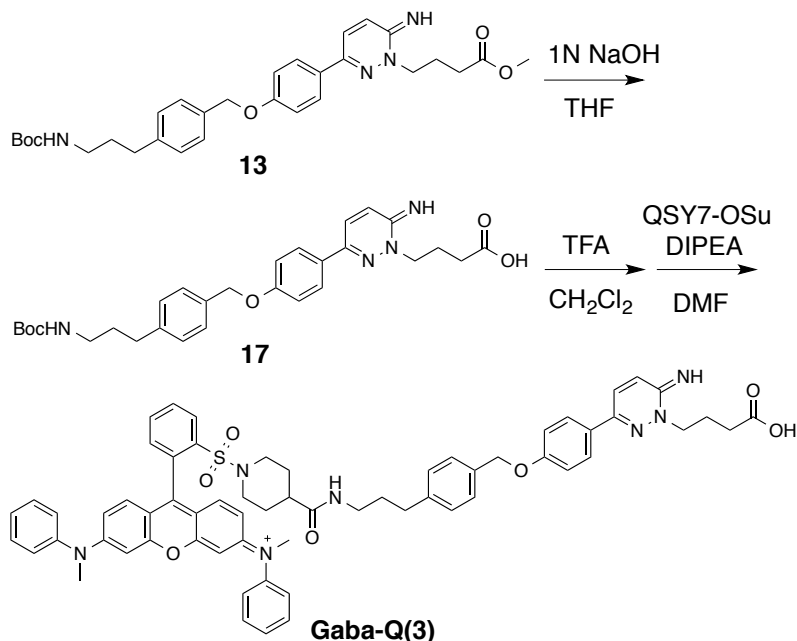
To a stirred solution of Oregon Green® 488 Carboxylic Acid, Succinimidyl Ester, 5-isomer (7 mg, 13.0 μ mol) in dry DMF (687 μ l) was added 2-(2-aminoethoxy)ethan-1-ol (10 μ l, 98.0 μ mol). The reaction mixture was allowed to stir for 1 h at room temperature. The mixture was purified by silica gel column chromatography (CHCl_3 only $\rightarrow \text{CHCl}_3 : \text{MeOH} = 3:1 \rightarrow \text{CHCl}_3 : \text{MeOH} : \text{H}_2\text{O} = 6:4:1$) to yield compound **16** (7 mg, 13.0 μ mol, quant.) as a yellow solid. $^1\text{H-NMR}$ (400 MHz, $\text{CDCl}_3 : \text{CD}_3\text{OD} = 1:1$): δ 8.53 (s, 1H), 8.17 (d, 1H, $J = 7.6$ Hz), 7.29 (d, 1H, $J = 8.0$ Hz), 6.79 (d, 2H, $J = 7.2$ Hz), 6.49 (d, 2H, $J = 10.8$ Hz), 3.66-3.72 (m, 6H), 3.59 (t, 2H, $J = 4.0$ Hz).

4-(3-(4-((4-(3-(2-(1-((2-(2-(2',7'-difluoro-3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-5-carboxamido)ethoxy)ethoxy)carbonyl)-1H-imidazol-4-yl)acetamido)propyl)benzyl)oxy)phenyl)-6-iminopyridazin-1(6H)-yl)butanoic acid (CGAM-Gaba(1))

To a stirred solution of compound **16** (118 mg, 0.24 mmol) in dry DMF (2.4 ml) was added Et_3N (100 μ l, 0.72 mmol) and di(N-succinimidyl) carbonate (91 mg, 0.35 mmol). The reaction mixture was allowed to stir for 5 h at room temperature. The solvent was evaporated. The residue was precipitated by ether : $\text{CHCl}_3 = 1:1$. The pellet was dissolved in dry DMF (1.5 ml). Then, compound **15** (41 mg, 0.075 mmol), DIPEA (40 μ l, 0.23 mmol) were added to the solution. The reaction mixture was allowed to stir for 3 h at room temperature. The mixture was purified by HPLC (mobile phase; CH_3CN (solvent A), 10 mM AcONH_4 aq. (solvent B)) to yield **CGAM-Gaba(1)** (2.8 μ mol, 3.8 %) as a yellow solid. $^1\text{H-NMR}$ (600 MHz, CD_3OD): δ 8.49 (s, 1H), 8.31 (m, 1H), 8.23 (m, 1H), 8.04 (m, 1H), 7.89 (m, 2H), 7.73 (m, 1H), 7.54 (m, 1H), 7.47 (s, 1H), 7.18-7.37 (m, 5H), 7.07 (m, 1H), 6.51-6.77 (m, 4H), 5.08 (s, 2H), 4.59 (m, 2H), 4.39 (m, 2H), 3.64-3.87 (m, 6H), 3.58 (s, 2H), 3.15 (m, 2H), 2.59 (m, 2H), 2.19-2.42 (m, 4H), 1.76 (m, 2H). $^{13}\text{C-NMR-dept}$ (150 MHz, CD_3OD): δ 138.72 (CH), 132.22 (CH), 129.71 (CH), 129.67 (CH), 129.19 (CH), 129.00 (CH), 128.98 (CH), 128.58 (CH), 126.36 (CH), 116.79 (CH), 116.33 (CH), 114.68 (CH), 105.83 (CH), 70.78 (CH_2), 70.45 (CH_2), 69.38 (CH_2), 68.57 (CH_2), 57.41 (CH_2), 41.19 (CH_2), 40.99 (CH_2), 35.87 (CH_2), 33.77 (CH_2), 32.46 (CH_2), 32.14 (CH_2), 23.2

1 (CH₂). HR-ESI MS: calcd for C₅₅H₄₉F₂N₇O₁₃ [M+H]⁺ = 1054.3435; obsd 1054.3424.

Synthesis of Gaba-Q(3).



4-(3-(4-((4-(3-((*tert*-butoxycarbonyl)amino)propyl)benzyl)oxy)phenyl)-6-iminopyridazin-1(6H)-yl)butanoic acid (17)

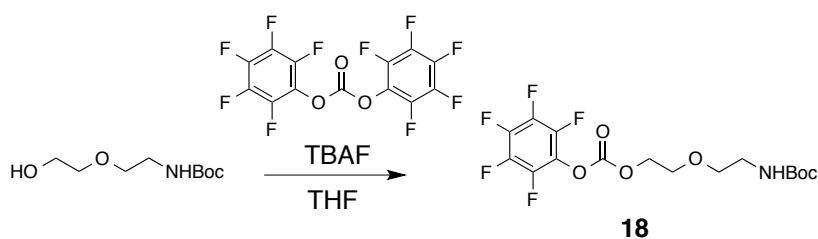
To a stirred solution of compound **13** (3 mg, 5.6 μmol) in THF (500 μl) and H₂O (400 μl) was added 1N NaOH (100 μl). The reaction mixture was allowed to stir for 3 h at 50 °C. The solution was neutralized with 1N HCl (100 μL). The solvent was evaporated to yield compound **17** (quant.) as a white solid. The crude compound **17** was used directly for the next step without further purification. ¹H-NMR (400 MHz, CD₃OD): δ 8.23 (d, 1H, *J* = 9.6 Hz), 7.94 (d, 2H, *J* = 8.4 Hz), 7.58 (d, 1H, *J* = 9.6 Hz), 7.38 (d, 2H, *J* = 8.4 Hz), 7.22 (d, 2H, *J* = 8.0 Hz), 7.16 (d, 2H, *J* = 8.8 Hz), 5.13 (s, 2H), 4.41 (t, 2H, *J* = 6.8 Hz), 3.06 (t, 2H, *J* = 6.4 Hz), 2.62 (t, 2H, *J* = 6.4 Hz), 2.56 (t, 2H, *J* = 6.4 Hz), 2.21 (t, 2H, *J* = 6.8 Hz), 1.77 (t, 2H, *J* = 7.2 Hz), 1.41 (s, 9H).

(*Z*)-N-(9-(2-((4-((3-(4-((4-(1-(3-carboxypropyl)-6-imino-1,6-dihydropyridazin-3-yl)phenoxy)methyl)phenyl)propyl)carbamoyl)piperidin-1-yl)sulfonyl)phenyl)-6-(methyl(phenyl)amino)-

3H-xanthen-3-ylidene)-N-methylbenzenaminium (Gaba-Q(4))

To a stirred solution of compound **17** (5.6 μmol) in dry CH_2Cl_2 (1 ml) was added TFA (0.5 ml). The reaction mixture was allowed to stir for 30 min at room temperature. After azeotropic removal of TFA with toluene, the residue was dissolved in dry DMF (200 μl). Then, QSY® 7 Carboxylic Acid, Succinimidyl Ester (2.5 mg, 3.2 μmol), DIPEA (15 μl , 84 μmol) were added to the solution. The reaction mixture was allowed to stir for 30 min at room temperature. The mixture was purified by HPLC (mobile phase; 0.1 % TFA CH_3CN (solvent A), 0.1 % TFA H_2O (solvent B)) to yield **Gaba-Q(3)** (0.5 μmol , 16 %) as a purple solid.

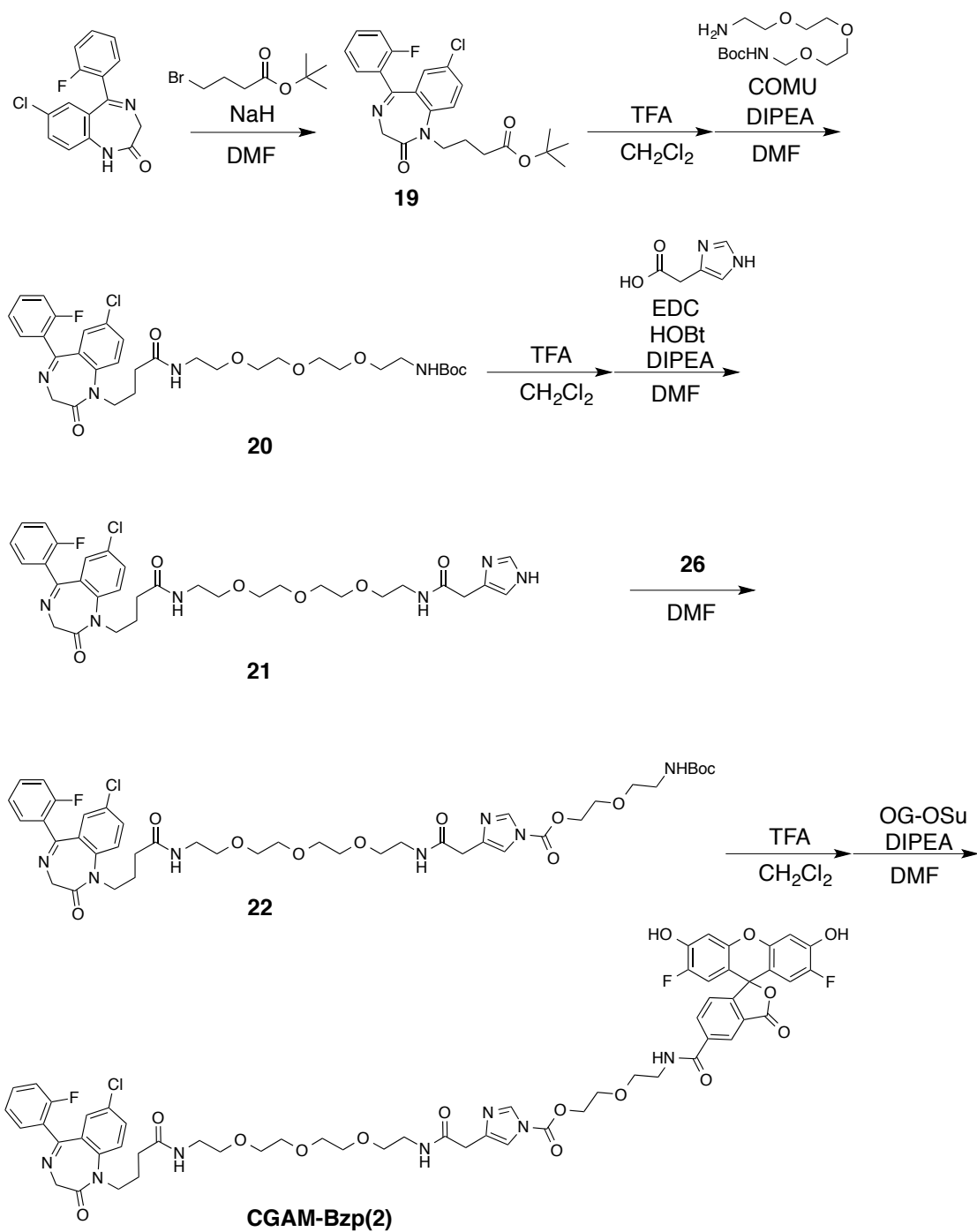
$^1\text{H-NMR}$ (600 MHz, CD_3OD): δ 8.26 (d, 1H, $J = 9.6$ Hz), 8.15-8.17 (m, 2H), 7.93 (d, 2H, $J = 9.0$ Hz), 7.86-7.88 (m, 3H), 7.54-7.59 (m, 5H), 7.44-7.49 (m, 4H), 7.34-7.39 (m, 4H), 7.20 (d 2H, $J = 7.8$ Hz), 7.13 (d, 2H, $J = 9.0$ Hz), 7.09 (d, 2H, $J = 9.6$ Hz), 6.95 (d, 2H, $J = 2.4$ Hz), 6.90 (dd, 2H, $J = 2.4, 9.6$ Hz), 5.12 (s, 2H), 4.43 (t, 2H, $J = 7.2$ Hz), 3.60 (s, 6H), 3.17 (t, 2H, $J = 6.6$ Hz), 2.63 (t, 2H, $J = 7.2$ Hz), 2.55 (t, 2H, $J = 12.0$ Hz), 2.50 (t, 2H, $J = 6.6$ Hz), 2.15-2.21 (m, 3H), 1.80 (t, 2H, $J = 7.2$ Hz), 1.61 (m, 4H), 1.38-1.40 (m, 2H). $^{13}\text{C-NMR}$ (150 MHz, CD_3OD): δ 177.42, 176.60, 162.74, 159.28, 159.15, 158.61, 153.89, 151.99, 146.10, 143.03, 140.05, 135.74, 134.25, 132.94, 132.54, 132.52, 132.29, 132.06, 131.75, 131.12, 129.65, 129.64, 129.34, 128.91, 127.81, 126.73, 126.54, 117.17, 116.63, 116.59, 98.82, 71.09, 57.07, 45.91, 42.78, 41.95, 39.98, 33.93, 32.14, 31.64, 29.25, 23.06. HR-ESI MS: calcd for $\text{C}_{63}\text{H}_{62}\text{N}_7\text{O}_7\text{S}$ $[\text{M}]^+ = 1060.4426$; obsd 1060.4408.

Synthesis of 18.

***Tert*-butyl (2-(2-(((perfluorophenoxy)carbonyl)oxy)ethoxy)ethyl)carbamate (**18**)**

To a stirred solution of *tert*-butyl (2-(2-hydroxyethoxy)ethyl)carbamate (500 mg, 2.44 mmol) in THF (24 ml) was added bis(perfluorophenyl) carbonate (1.15 g, 2.93 mmol), TBAF (190 mg, 0.73 mmol). The reaction mixture was allowed to stir for 24 h at room temperature. The solution was diluted with CHCl₃ and washed with 1N NaOH. The organic layer was dried over Na₂SO₄, filtered, evaporated. The residue was purified by silica gel column chromatography (Hexane : AcOEt = 10:1 → Hexane : AcOEt = 2:1) to yield Compound **18** (653 mg, 1.579 mmol, 64 %) as a clear oil. ¹H-NMR (400 MHz, CDCl₃): δ 4.90 (s, 1H), 4.47 (m, 2H), 3.79 (m, 2H), 3.59 (t, 2H, *J* = 5.6 Hz), 3.36 (m, 2H), 1.46 (s, 9H).

Synthesis of CGAM-Bzp(2).



***Tert*-butyl 4-(7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-1-yl)butanoate (19)**

To a stirred solution of 7-chloro-5-(2-fluorophenyl)-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one (200 mg, 0.69 mmol) in dry DMF (700 μ l) was NaH (38 mg, 1.0 mmol) at 0 °C. The reaction mixture was allowed to stir for 5 min at 0 °C. Then, *tert*-butyl 4-bromobutanoate (185 mg, 0.83 mmol) in dry DMF (700 μ l) were added to the solution. The reaction mixture was allowed to stir for 1 h at room temperature. The solution was diluted with CHCl₃ and washed with H₂O. The organic layer was dried over Na₂SO₄, filtered, evaporated. The residue was purified by silica gel column chromatography (Hexane : AcOEt = 4:1 $\rightarrow$ Hexane : AcOEt = 2:1) to yield Compound **19** (125 mg, 0.29 mmol, 42 %) as a clear solid. ¹H-NMR (400 MHz, CDCl₃): δ 7.67 (t, 1H, *J* = 8.4 Hz), 7.49 (m, 2H), 7.40 (d, 1H, *J* = 8.8 Hz), 7.29 (m, 1H), 7.17 (s, 1H), 7.07 (t, 1H, *J* = 9.6 Hz), 4.87 (d, 1H, *J* = 10.4 Hz), 4.40 (m, 1H), 3.78 (d, 1H, *J* = 10.4 Hz), 3.67 (m, 1H), 2.15 (m, 2H), 1.86 (m, 1H), 1.72 (m, 1H), 1.39 (s, 9H).

***Tert*-butyl (16-(7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-1-yl)-13-oxo-3,6,9-trioxa-12-azahexadecyl)carbamate (20)**

To a stirred solution of compound **19** (18 mg, 0.042 mmol) in dry CH₂Cl₂ (1 ml) was added TFA (0.5 ml). The reaction mixture was allowed to stir for 1 h at room temperature. After azeotropic removal of TFA with toluene, the residue was dissolved in dry DMF (420 μ l). Then, *tert*-butyl ((2-(2-(2-aminoethoxy)ethoxy)ethoxy)methyl)carbamate (13 mg, 0.063 mmol), COMU (27 mg, 0.063 mmol), DIPEA (73 μ l, 0.42 mmol) were added to the solution. The reaction mixture was allowed to stir for 1 h at room temperature. The solution was diluted with CHCl₃ and washed with 1N NaOH. The organic layer was dried over Na₂SO₄, filtered, evaporated. The residue was purified by silica gel column chromatography (Hexane : AcOEt = 2:1 $\rightarrow$ CHCl₃ : MeOH = 10:1) to yield compound **20** (24 mg, 0.037 mmol, 88 %) as a clear solid. ¹H-NMR (400 MHz, CDCl₃): δ 7.56-7.67 (m, 4H), 7.34 (m, 1H), 7.18 (m, 1H), 7.14 (s, 1H), 4.69 (d, 1H, *J* = 10.8 Hz), 4.34 (m,

1H), 3.88 (d, 1H, $J = 10.8$ Hz), 3.79 (m, 1H), 3.18-3.67 (m, 16H), 2.14 (m, 2H), 1.85 (m, 1H), 1.70 (m, 1H), 1.42 (s, 9H).

N-(1-(1H-imidazol-4-yl)-2-oxo-6,9,12-trioxa-3-azatetradecan-14-yl)-4-(7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-1-yl)butanamide (21)

To a stirred solution of compound **20** (24 mg, 0.037 mmol) in dry CH_2Cl_2 (1 ml) was added TFA (0.5 ml). The reaction mixture was allowed to stir for 1 h at room temperature. After azeotropic removal of TFA with toluene, the residue was dissolved in dry DMF (370 μl). Then, 2-(1H-imidazol-4-yl)acetic acid (6 mg, 0.055 mmol), EDC (10 mg, 0.055 mmol), HOBT (8 mg, 0.055 mmol), DIPEA (64 μl , 0.37 mmol) were added to the solution. The reaction mixture was allowed to stir for 1 h at room temperature. The mixture was purified by silica gel column chromatography (CHCl_3 : MeOH : NH_4OH aq. = 10:1:1) to yield compound **21** (18 mg, 0.027 mmol, 74 %) as a clear solid. $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 7.62 (t, 1H, $J = 6.4$ Hz), 7.53 (s, 1H), 7.41-7.49 (m, 4H), 7.12 (s, 1H), 7.06 (t, 1H, $J = 9.2$ Hz), 6.84 (s, 1H), 4.81 (d, 1H, $J = 10.4$ Hz), 4.29 (m, 1H), 3.77 (d, 1H, $J = 10.4$ Hz), 3.67 (m, 1H), 3.51 (s, 1H), 2.05 (m, 2H), 1.91 (m, 1H), 1.65 (m, 1H).

2-(2-((*tert*-butoxycarbonyl)amino)ethoxy)ethyl 4-(19-(7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-1-yl)-2,16-dioxo-6,9,12-trioxa-3,15-diazanonadecyl)-1H-imidazole-1-carboxylate (22)

To a stirred solution of compound **21** (18 mg, 0.027 mmol) in dry DMF (270 μl) was added compound **18** (17 mg, 0.04 mmol). The reaction mixture was allowed to stir for 1 h at room temperature. The reaction mixture was purified by silica gel column chromatography (CHCl_3 only $\rightarrow \text{CHCl}_3$: MeOH = 10:1) to yield compound **22** (15 mg, 0.017 mmol, 63 %) as a clear solid. $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 8.10 (s, 1H), 7.62 (t, 1H, $J = 7.6$ Hz), 7.41-7.49 (m, 3H), 7.35 (s, 1H), 7.26 (m, 1H), 7.13 (s, 1H), 7.06 (t, 1H, $J = 9.2$ Hz), 4.82 (d, 1H, $J = 10.8$ Hz), 4.52 (m, 2H),

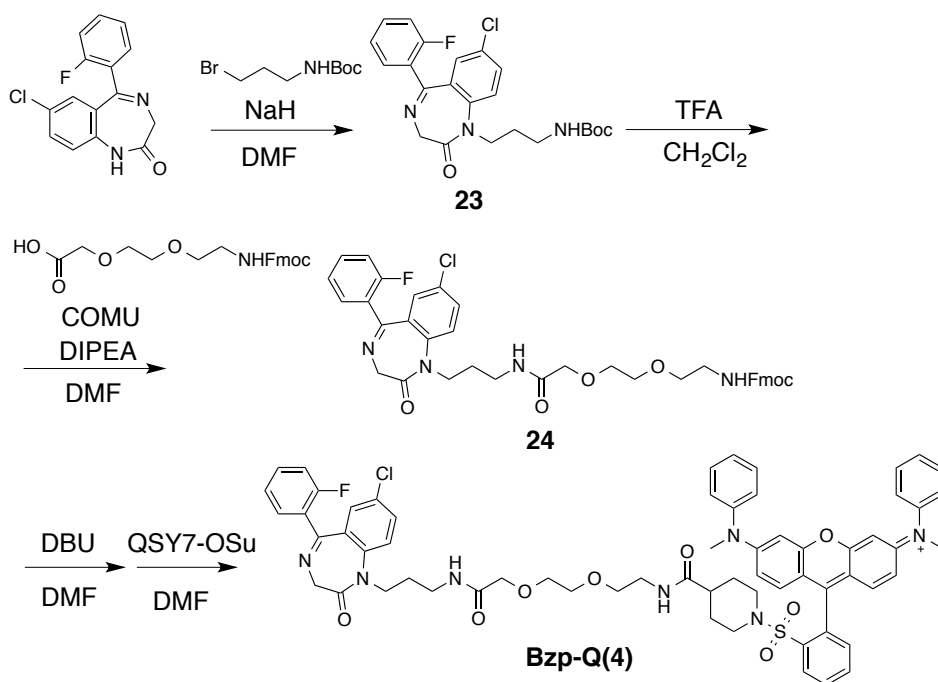
4.32 (m, 1H), 3.30-3.77 (m, 28H), 2.09 (m, 2H), 1.94 (m, 1H), 1.70 (m, 1H), 1.42 (s, 9H).

2-(2-(2',7'-difluoro-3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-5-carboxamido)ethoxy)ethyl 4-(19-(7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-1-yl)-2,16-dioxo-6,9,12-trioxa-3,15-diazanonadecyl)-1H-imidazole-1-carboxylate (CGAM-Bzp(2))

To a stirred solution of compound **22** (1 mg, 1.1 μ mol) in dry CH_2Cl_2 (0.5 ml) was added TFA (0.5 ml). The reaction mixture was allowed to stir for 1 h at room temperature. After azeotropic removal of TFA with toluene, the residue was dissolved in dry DMF (100 μ l). Then, Oregon Green® 488 Carboxylic Acid, Succinimidyl Ester, 5-isomer (1 mg, 2.0 μ mol), DIPEA (20 μ l, 0.11 mmol) were added to the solution. The reaction mixture was allowed to stir for 1 h at room temperature. The mixture was purified by HPLC (mobile phase; CH_3CN (solvent A), 10 mM AcONH_4 aq. (solvent B)) to yield **CGAM-Bzp(2)** (0.8 mg, 0.7 μ mol, 64 %) as a yellow solid.

$^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 8.44 (s, 1H), 8.11-8.17 (m, 2H), 7.89 (s, 1H), 7.57-7.62 (m, 3H), 7.45 (s, 1H), 7.32 (m, 2H), 7.13-7.18 (m, 2H), 6.80 (d, 2H, $J = 7.2$ Hz), 6.49 (d, 2H, $J = 5.8$ Hz), 4.67 (d, 1H, $J = 10.8$ Hz), 4.58 (m, 2H), 4.32 (m, 1H), 3.45-3.87 (m, 28H), 2.12 (m, 2H), 1.82 (m, 1H), 1.66 (m, 1H). $^{13}\text{C-NMR-dept}$ (150 MHz, CD_3OD): δ 138.75 (CH), 134.50 (CH), 134.45 (CH), 133.47 (CH), 132.75 (CH), 129.55 (CH), 126.15 (CH), 126.12 (CH), 125.84 (CH), 117.51 (CH), 117.37 (CH), 116.94 (CH), 114.83 (CH), 106.05 (CH), 71.64 (CH_2), 71.63 (CH_2), 71.24 (CH_2), 71.21 (CH_2), 70.55 (CH_2), 70.47 (CH_2), 69.51 (CH_2), 68.62 (CH_2), 57.83 (CH_2), 47.07 (CH_2), 41.18 (CH_2), 40.64 (CH_2), 40.41 (CH_2), 35.85 (CH_2), 33.49 (CH_2), 24.90 (CH_2).

HR-ESI MS: calcd for $\text{C}_{58}\text{H}_{55}\text{ClF}_3\text{N}_7\text{O}_{15}$ $[\text{M}+\text{H}]^+ = 1182.3475$: obsd 1182.3497.

Synthesis of **Bzp-Q(4)**.***Tert*-butyl****(3-(7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-1-yl)propyl)carbamate (23)**

To a stirred solution of 7-chloro-5-(2-fluorophenyl)-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one (35 mg, 0.12 mmol) in dry DMF (120 μ l) was added NaH (10 mg, 0.24 mmol) at 0 °C. The reaction mixture was allowed to stir for 5 min at 0 °C. Then, *tert*-butyl (3-bromopropyl)carbamate (55 mg, 0.24 mmol) in dry DMF (120 μ l) was added to the solution. The reaction mixture was allowed to stir for 3 h at room temperature. The solution was diluted with CHCl₃ and washed with H₂O. The organic layer was dried over Na₂SO₄, filtered, evaporated. The residue was purified by silica gel column chromatography (Hexane : AcOEt = 4:1 $\rightarrow$ Hexane : AcOEt = 2:1) to yield compound **23** (23 mg, 51.6 μ mol, 43 %) as a clear solid.

¹H-NMR (400 MHz, CDCl₃): δ 7.67 (t, 1H, *J* = 7.6 Hz), 7.46-7.52 (m, 2H), 7.39 (d, 1H, *J* = 8.8 Hz), 7.17 (m, 1H), 7.04-7.09 (m, 2H), 4.91 (brs, 1H), 4.87 (d, 1H, *J* = 10.4 Hz), 4.53 (m, 1H), 3.78 (d, 1H, *J* = 10.4 Hz), 3.64 (m, 1H), 3.18 (m, 2H), 1.72 (m, 1H), 1.53 (m, 1H), 1.44 (s, 9H).

(9H-fluoren-9-yl)methyl**(2-(2-(2-((3-(7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-1-yl)propyl)amino)-2-oxoethoxy)ethoxy)ethyl)carbamate (24)**

To a stirred solution of compound **23** (12 mg, 27.0 μmol) in dry CH_2Cl_2 (1 ml) was added TFA (1 ml). The reaction mixture was allowed to stir for 1 h at room temperature. After azeotropic removal of TFA with toluene, the residue was dissolved in dry DMF (270 μl). Then, 1-(9H-fluoren-9-yl)-3-oxo-2,7,10-trioxa-4-azadodecan-12-oic acid (15 mg, 40.0 μmol), COMU (17 mg, 40.0 μmol), DIPEA (23 μl , 0.13 mmol) were added to the solution. The reaction mixture was allowed to stir for 30 min at room temperature. The mixture was purified by silica gel column chromatography (CHCl_3 only $\rightarrow \text{CHCl}_3 : \text{MeOH} = 10:1$) to yield compound **24** (10 mg, 14.0 μmol , 48 %) as a clear solid. $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 7.72 (m, 2H), 7.58 (m, 3H), 7.46 (m, 2H), 7.21-7.41 (m, 6H), 7.14 (s, 1H), 7.05 (t, 1H, $J = 8.4$ Hz), 5.98 (brs, 1H), 4.83 (d, 1H, $J = 10.0$ Hz), 4.51 (m, 1H), 4.31-4.42 (m, 4H), 4.21 (m, 1H), 3.97 (d, 1H, $J = 10.0$ Hz), 3.58-3.67 (m, 6H), 3.39-3.50 (m, 3H), 3.24 (m, 2H), 1.73 (m, 1H), 1.52 (m, 1H).

(Z)-N-(9-(2-((4-((2-(2-((3-(7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-1-yl)propyl)amino)-2-oxoethoxy)ethoxy)ethyl)carbamoyl)piperidin-1-yl)sulfonyl)phenyl)-6-(methyl(phenyl)amino)-3H-xanthen-3-ylidene)-N-methylbenzenaminium (Bzp-Q(4))

To a stirred solution of compound **24** (3.0 mg, 4.2 μmol) in dry DMF (200 μl) was added DBU (20 μl). The reaction mixture was allowed to stir for 1 h at room temperature. Then, QSY® 7 Carboxylic Acid, Succinimidyl Ester (2.5 mg, 3.2 μmol) was added to the solution. The reaction mixture was allowed to stir for 30 min at room temperature. The mixture was purified by HPLC (mobile phase; 0.1 % TFA CH_3CN (solvent A), 0.1 % TFA H_2O (solvent B)) to yield **Bzp-Q(4)** (0.5 μmol , 48 %) as a purple solid. $^1\text{H-NMR}$ (600 MHz, CD_3OD): δ 8.13 (m, 1H), 7.86 (m, 2H),

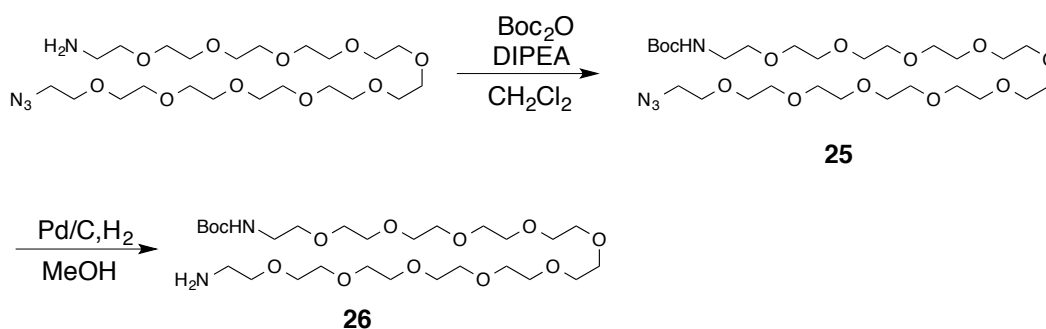
7.65 (m, 1H), 7.59 (s, 2H), 7.56 (m, 4H), 7.44-7.48 (m, 3H), 7.37 (m, 4H), 7.32 (m, 1H), 7.13 (m, 2H), 7.09 (d, 2H, $J = 9.6$ Hz), 6.95 (s, 2H), 6.89 (d, 2H, $J = 9.6$ Hz), 4.66 (d, 1H, $J = 10.8$ Hz), 4.39 (m, 1H), 3.92 (s, 2H), 3.85 (d, 1H, $J = 10.8$ Hz), 3.75 (m, 1H), 3.60-3.65 (m, 10H), 3.51 (t, 2H, $J = 5.9$ Hz), 3.19-3.25 (m, 2H), 2.52 (t, 2H, $J = 12.0$ Hz), 2.19 (m, 2H), 1.29-1.44 (m, 6H).

^{13}C -NMR (150 MHz, CD_3OD): δ 176.78, 172.63, 170.39, 168.12, 159.28, 159.14, 158.67, 146.11, 141.06, 140.15, 134.

21, 134.19, 134.13, 133.85, 133.24, 132.95, 132.55, 132.54, 132.51, 132.29, 132.07, 131.76, 131.63, 131.02, 129.64, 129.40, 127.81, 125.96, 125.94, 125.70, 117.30, 117.19, 117.18, 117.16, 116.59, 98.83, 71.98, 71.30, 71.29, 70.55, 57.80, 45.98, 45.28, 42.81, 41.96, 40.20, 36.94, 29.28, 28.8

8. HR-ESI MS: calcd for $\text{C}_{63}\text{H}_{62}\text{ClFN}_7\text{O}_8\text{S}$ $[\text{M}]^+ = 1130.4048$: obsd 1130.4034.

Synthesis of **26**.

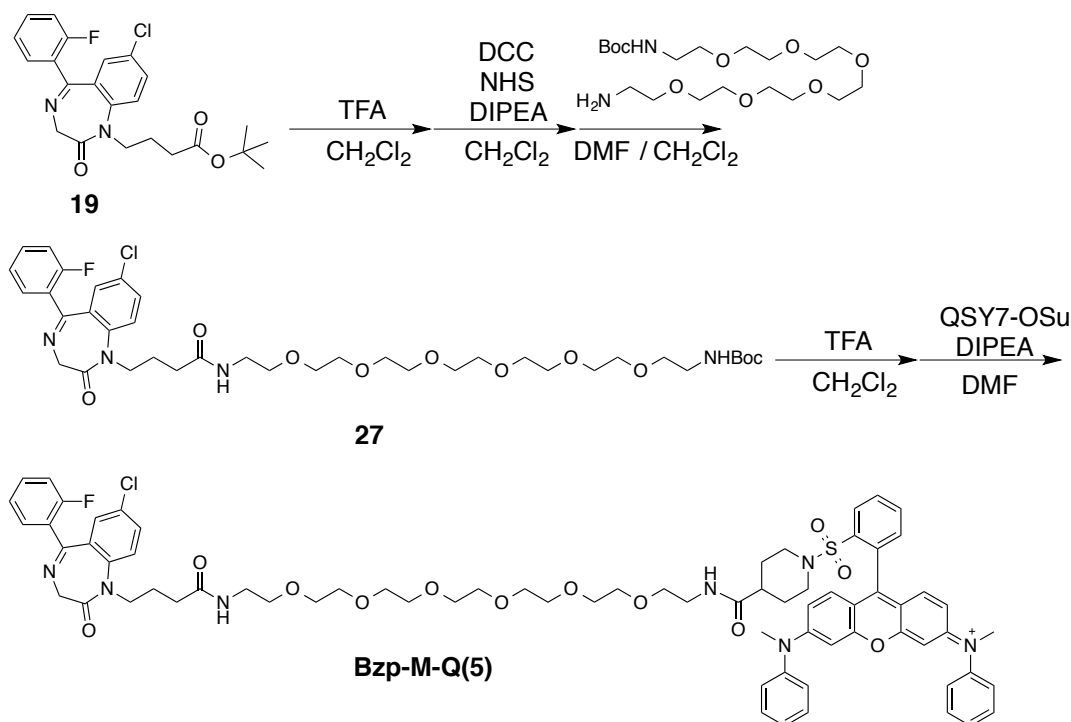


***Tert*-butyl (32-azido-3,6,9,12,15,18,21,24,27,30-decaoxadotriacontyl)carbamate (**25**)**

To a stirred solution of 32-azido-3,6,9,12,15,18,21,24,27,30-decaoxadotriacontan-1-amine (127 mg, 0.36 mmol) in THF (3.6 ml) was added Boc_2O (94 mg, 0.43 mmol), DIPEA (63 μl , 0.36 mmol). The reaction mixture was allowed to stir for 1 h at room temperature. The solution was purified by silica gel column chromatography (Hexane : AcOEt = 2:1 $\rightarrow$ CHCl_3 only $\rightarrow$ CHCl_3 : MeOH = 10:1) to yield compound **25** (145 mg, 0.32 mmol, 89 %) as a clear oil. ^1H -NMR (400 MHz, CDCl_3): δ 3.89 (m, 2H), 3.74 (m, 2H), 3.62-3.70 (m, 24H), 3.54 (m, 2H), 3.31 (m, 2H), 3.14 (m, 2H), 1.44 (s, 9H).

***Tert*-butyl (32-amino-3,6,9,12,15,18,21,24,27,30-decaoxadotriacontyl)carbamate (26)**

To a stirred solution of compound **25** (30 mg, 0.066 mmol) in MeOH (1.5 ml) was added 10 % *palladium-carbon* (3 mg) at room temperature. The reaction mixture was allowed to stir for 1 h under hydrogen atmosphere. The solution was filtered by Celite and evaporated to give compound **26** as a clear oil. The crude compound **26** was used directly for the next step without further purification.

Synthesis of Bzp-M-Q(5)***Tert*-butyl (25-(7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-1-yl)-22-oxo-3,6,9,12,15,18-hexaoxa-21-azapentacosyl)carbamate (27)**

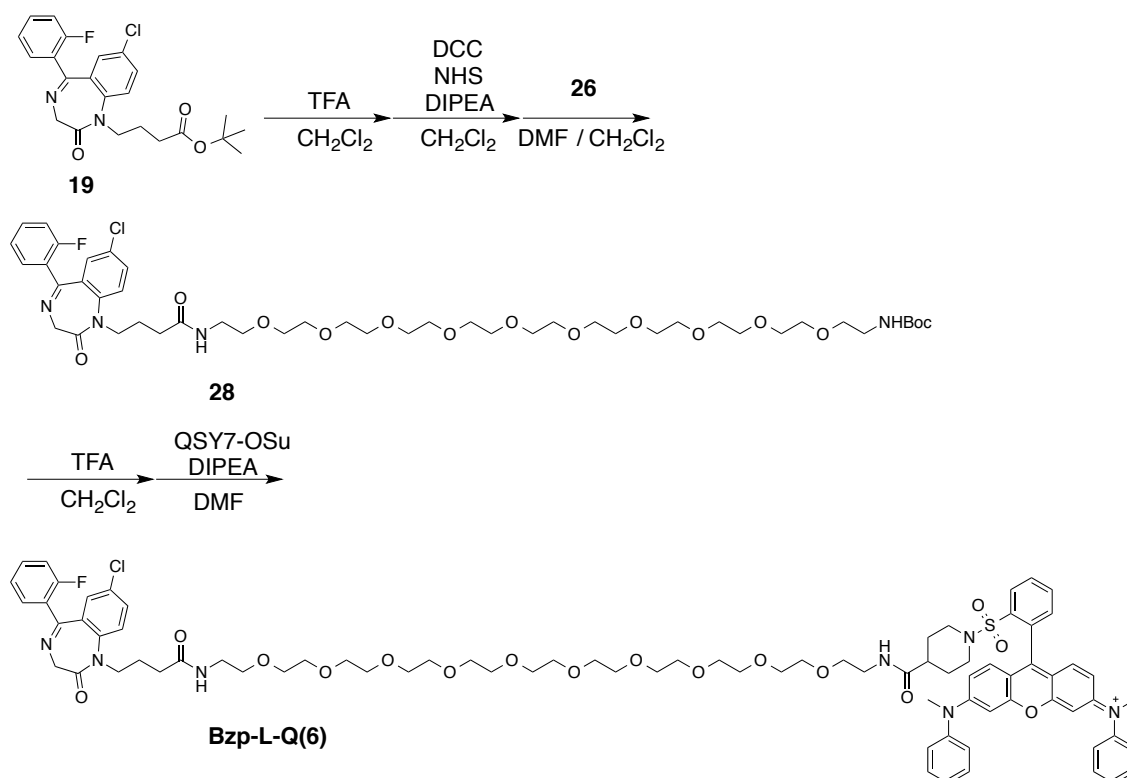
To a stirred solution of compound **19** (29 mg, 0.066 mmol) in dry CH₂Cl₂ (1 ml) was added TFA (0.5 ml). The reaction mixture was allowed to stir for 30 min at room temperature. After azeotropic removal of TFA with toluene, the residue was dissolved in dry CH₂Cl₂ (1.3 ml). Then, DCC (21 mg, 0.1 mmol), NHS (10 mg, 0.1 mmol), DIPEA (34 μ l, 0.2 mmol) were added to the

solution. The reaction mixture was allowed to stir for 1.5 h at room temperature. Then, *tert*-butyl (20-amino-3,6,9,12,15,18-hexaoxaicosyl)carbamate (0.066 mmol) in dry DMF (400 μ l) was added to the solution. The reaction mixture was allowed to stir for 1 h at room temperature. The solution was purified by silica gel column chromatography (CHCl_3 only $\rightarrow$ CHCl_3 : MeOH= 20:1) to yield Compound **27** (26 mg, 0.033 mmol, 50 %) as a clear oil.

(Z)-N-(9-(2-((4-((25-(7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-1-yl)-22-oxo-3,6,9,12,15,18-hexaoxa-21-azapentacosyl)carbamoyl)piperidin-1-yl)sulfonyl)phenyl)-6-(methyl(phenyl)amino)-3H-xanthen-3-ylidene)-N-methylbenzenaminium (Bzp-M-Q(5))

To a stirred solution of compound **27** (4 mg, 5.3 μ mol) in dry CH_2Cl_2 (1 ml) was added TFA (0.5 ml). The reaction mixture was allowed to stir for 30 min at room temperature. After azeotropic removal of TFA with toluene, the residue was dissolved in dry DMF (300 μ l). Then, QSY7-OSu (2 mg, 2.6 μ mol), DIPEA (30 μ l) were added to the solution. The reaction mixture was allowed to stir for 30 min at room temperature. The solution was purified by HPLC (mobile phase; 0.1 % TFA CH_3CN (solvent A), 0.1 % TFA H_2O (solvent B)) to yield **Bzp-M-Q(5)** (1.8 mg, 1.36 μ mol, 54 %) as a purple solid. HR-ESI MS: calcd for $\text{C}_{72}\text{H}_{80}\text{ClFN}_7\text{O}_{12}\text{S}$ $[\text{M}]^+ = 1320.5253$: obsd 1320.5233.

Synthesis of Bzp-L-Q(6)



Tert-butyl (37-(7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-1-yl)-34-oxo-3,6,9,12,15,18,21,24,27,30-decaoxa-33-azaheptatriacontyl)carbamate (**28**)

To a stirred solution of compound **19** (10 mg, 0.023 mmol) in dry CH_2Cl_2 (1 ml) was added TFA (0.5 ml). The reaction mixture was allowed to stir for 30 min at room temperature. After azeotropic removal of TFA with toluene, the residue was dissolved in dry CH_2Cl_2 (460 μl). Then, DCC (7 mg, 0.035 mmol), NHS (3.5 mg, 0.035 mmol), DIPEA (18 μl , 0.105 mmol) were added to the solution. The reaction mixture was allowed to stir for 1.5 h at room temperature. Then, compound **26** (0.066 mmol) in dry DMF (200 μl) was added to the solution. The reaction mixture was allowed to stir for 1 h at room temperature. The solution was purified by silica gel column chromatography (CHCl_3 only $\rightarrow \text{CHCl}_3$: MeOH = 20:1) to yield compound **28** (8.3 mg, 8.67 mmol, 38 %) as a clear oil.

(*Z*)-N-(9-(2-((4-((37-(7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin

n-1-yl)-34-oxo-3,6,9,12,15,18,21,24,27,30-decaoxa-33-azaheptatriacontyl)carbamoyl)piperidin-1-yl)sulfonyl)phenyl)-6-(methyl(phenyl)amino)-3H-xanthen-3-ylidene)-N-methylbenzenaminium (Bzp-L-Q(6))

To a stirred solution of compound **28** (3 mg, 3.0 μmol) in dry CH_2Cl_2 (1 ml) was added TFA (0.5 ml). The reaction mixture was allowed to stir for 30 min at room temperature. After azeotropic removal of TFA with toluene, the residue was dissolved in dry DMF (300 μl). Then, QSY7-OSu (1 mg, 1.5 μmol), DIPEA (30 μl) were added to the solution. The reaction mixture was allowed to stir for 30 min at room temperature. The solution was purified by HPLC (mobile phase; 0.1 % TFA CH_3CN (solvent A), 0.1 % TFA H_2O (solvent B)) to yield **Bzp-L-Q(6)** (0.9 mg, 0.6 μmol , 40 %) as a purple solid. HR-ESI MS: calcd for $\text{C}_{80}\text{H}_{96}\text{ClFN}_7\text{O}_{16}\text{S}$ $[\text{M}+\text{H}]^+ = 1496.6301$; obsd 1496.6272.

General methods for biological experiments

Construction of expression plasmids for $\alpha 1/\beta 3/\gamma 2$ GABA_ARs and $\alpha 3, \alpha 5$ GABA_ARs

For construction of pCAGGS($\alpha 1$ GABA_AR), pCAGGS($\beta 3$ GABA_AR), and pCAGGS($\gamma 2$ GABA_AR), cDNA fragments of mouse GABA_AR $\alpha 1$, $\beta 3$, and $\gamma 2$ subunit were obtained from PRK5($\alpha 1$)⁴³, PRK5($\beta 3$)⁴⁴, PRK5($\gamma 2$)⁴⁴, respectively, by applying a PCR-based approach. The cDNA fragments were subsequently subcloned into pCAGGS vector⁴⁵ to obtain pCAGGS($\alpha 1$ GABA_AR), pCAGGS($\beta 3$ GABA_AR), and pCAGGS($\gamma 2$ GABA_AR). PRK5($\alpha 1$), PRK5($\beta 3$), and PRK5($\gamma 2$) were kindly gifted from Dr. S. Moss. For construction of pCAGGS($\alpha 3$ GABA_AR), pCAGGS($\alpha 5$ GABA_AR), cDNA fragments of mouse GABA_AR $\alpha 3$ and $\alpha 5$ subunit were obtained from Mouse Brain Marathon®-Ready cDNA, by applying a PCR-based approach. The cDNA fragments were subsequently subcloned into pCAGGS vector to obtain pCAGGS($\alpha 3$ GABA_AR), pCAGGS($\alpha 5$ GABA_AR). All cDNA sequences were verified by DNA sequencing.

cDNA expression in HEK293T cells.

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 30 units ml⁻¹ penicillin and 30 units ml⁻¹ streptomycin under 95% air-5% CO₂ atmosphere at 37°C. One day before transfection, HEK293T cells (2.0×10^5 cells) were plated on 3.5-cm dishes (BD Falcon). For expression of $\alpha 1/\beta 3/\gamma 2$, $\alpha 3/\beta 3/\gamma 2$, or $\alpha 5/\beta 3/\gamma 2$ GABA_ARs, the cells were transfected with 0.7 μ g of each expression vectors for α , β , and γ subunit (total 2.1 μ g) and 0.3 μ g of pmCherry-N1 (Clontech) as a transfection marker using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. For expression of $\alpha 1/\beta 3$ GABA_ARs, the cells were transfected with 0.7 μ g of each expression vectors for $\alpha 1$ and $\beta 3$ subunit (total 1.4 μ g) and 0.3 μ g of pmCherry-N1. As a control experiment, the empty vector (pCAGGS) was transfected.

Chemical labeling of GABA_ARs in HEK293T cells

HEK293T cells (2.0×10^5 cells) were transiently transfected as described in 'cDNA expression in HEK293T cells'. After 48 h of transfection, the medium was replaced by serum-free DMEM

containing **CGAM** reagents (**CGAM-Gaba** or **CGAM-Bzp**) in the presence or absence of each inhibitor at 37 °C for each period. After removal of the medium, the cells were washed twice with PBS. The cells were lysed with RIPA buffer (25 mM Tris/HCl, 150 mM NaCl, 1% NP-40, 0.25% deoxycholate (DOC), 0.1% SDS, pH 7.4) containing protease inhibitor (Calbiochem, 39134), and rotated at 4 °C for 30 min. The lysate was centrifuged ($10,000 \times g$, 10 min, 4 °C). The supernatant was mixed with 5 $\times$ SDS-PAGE loading buffer (325 mM Tris-HCl, 15% SDS, 20% sucrose, 0.5 M DTT, and 0.02% BPB, pH 6.8) and incubated for 1 h at room temperature. The samples were applied to SDS-PAGE and electrotransferred onto immune-blot PVDF membranes (Bio-rad), followed by blocking with 5% non-fat dry milk in TBS containing 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO). The membranes were stained with rabbit anti-fluorescein/Oregon green antibody (Abcam, ab19491), rabbit anti-GABA_AR α 1 antibody (Millipore, 06-868), rabbit anti-GABA_AR β 3 antibody (Rockland, 600-401-C79), rabbit anti-GABA_AR γ 2 antibody (Rockland, 612-401-C81), or rabbit anti- β -tubulin antibody (Abcam, ab6046) followed by a HRP-conjugated goat anti-rabbit IgG (Santa cruz, SC-2004). The membranes were developed with ECL prime western blotting detection system (GE Healthcare).

Confocal live imaging of HEK293T cells labeled by CGAM reagents

HEK293T cells (2.0×10^5 cells) were transiently transfected as described in 'cDNA expression in HEK293T cells'. After 24 h of transfection, the cells were dissociated by pipetting and re-seeded on glass-bottom dishes pretreated with poly-L-lysine. After 24 h of the re-seeding, the medium was replaced with serum-free DMEM containing **CGAM-Gaba** or **CGAM-Bzp** in the presence or absence of each inhibitor at 37 °C for each period. After removal of the medium, the cells were washed twice with serum-free DMEM. Fluorescent imaging of the cells was performed using a confocal laser scanning microscopy (Olympus, FLUOVIEW FV1000) equipped with a 60 $\times$, NA = 1.40 oil objective, and a GaAsP detector. Fluorescence images were acquired using the 488 nm excitation derived from an Ar laser for OG, and 543 nm derived from a HeNe laser for mCherry.

Subunit specificity for GABA_AR labeling using CGAM reagents

HEK293T cells (2.0×10^5 cells) were incubated on 35-mm dishes (BD Falcon) at 37 °C. After 18

h, the cells were transiently transfected with varied compositions of the GABA_AR subunits (a single subunit of $\alpha 1$, $\beta 3$, $\gamma 2$ (0.7 μg), two subunits of $\alpha 1/\beta 3$, $\beta 3/\gamma 2$, $\alpha 1/\gamma 2$ (total 1.4 μg), or three subunits of $\alpha 1/\beta 3/\gamma 2$ (total 2.1 μg)). After 48 h of the transfection, the medium was replaced with serum-free DMEM containing **CGAM-Gaba** or **CGAM-Bzp** at 37 °C for each period. After removal of the medium, the cells were washed twice with PBS. The cells were lysed with RIPA buffer (25 mM Tris/HCl, 150 mM NaCl, 1% NP-40, 0.25% DOC, 0.1% SDS, pH 7.4) containing protease inhibitor (Calbiochem, 39134), and rotated at 4 °C for 30 min. The lysate was centrifuged (10,000 $\times$ g, 10 min, 4 °C). The supernatant was mixed with 5 $\times$ SDS-PAGE loading buffer (325 mM Tris-HCl, 15% SDS, 20% sucrose, 0.5 M DTT, and 0.02% BPB, pH 6.8) and incubated for 1 h at room temperature. The samples were applied to SDS-PAGE and western blotting as described in the section of ‘Chemical labeling of GABA_ARs in HEK293T cells’.

Identification of labeling site of benzodiazepine binding site on GABA_ARs by peptide mass finger printing analysis

HEK293T cells (1.2 $\times$ 10⁶ cells for each dish) were plated on two 10-cm dishes (BD Falcon). After 18 h of plating, the cells were transiently transfected with $\alpha 1/\beta 3/\gamma 2$ GABA_ARs as described in ‘cDNA expression in HEK293T cells’. After 48 h of transfection, the medium was replaced by serum-free DMEM containing 3 μM **CGAM-Bzp** at 37 °C for 4 h. After removal of the medium, the cells were washed twice with PBS. The cells were lysed with RIPA buffer (25 mM Tris/HCl, 150 mM NaCl, 1% NP-40, 0.25% deoxycholate (DOC), 0.1% SDS, pH7.4) containing protease inhibitor (Calbiochem), and rotated at 4 °C for 30 min. After centrifugation of the lysate (10,000 $\times$ g, 5 min, 4 °C), the supernatant was incubated with anti-FI/OG antibody at 4 °C for 18 h, followed by addition of Dynabeads® Protein A (Thermo Fisher Scientific) and further incubation at 4 °C for two hours. The beads were washed three times with RIPA buffer. Protein was eluted by addition of 2 $\times$ SDS-PAGE sample buffer and boiling in heatblock for 5 min. The samples were applied to SDS-PAGE. The protein band corresponding to GABA_AR was excised from the in-gel fluorescence image. The excised gel was subjected to in-gel digestion using MS grade Trypsin (Thermo Fisher Scientific). All samples were analyzed by nanoflow reverse phase liquid chromatography followed by tandem MS, using a LTQ Orbitrap XL hybrid mass spectrometer

(Thermo Fisher Scientific). For LTQ-Orbitrap XL, a capillary reverse phase HPLC–MS/MS system composed of an Agilent 1100 series gradient pump equipped with Valco C2 valves with 150- μ m ports, and LTQ-Orbitrap XL hybrid mass spectrometer equipped with an XYZ nanoelectrospray ionization (NSI) source (AMR). Samples were automatically injected using PAL system (CTC analytics, Zwingen, Switzerland) into a peptide L-trap column OSD (5 μ m, AMR) attached to an injector valve for desalinating and concentrating peptides. After washing the trap with MS-grade water containing 0.1% trifluoroacetic acid and 2% acetonitrile (solvent C), the peptides were loaded into a nano HPLC capillary column (C18-packed with the gel particle size of 3 μ m, 0.1 $\times$ 150 mm, Nikkyo Technos, Tokyo Japan) by switching the valve. The eluents used were A, 100% water containing 0.1% formic acid, and B, 80% acetonitrile containing 0.1% formic acid. The column was developed with the concentration gradient of acetonitrile as follows: from 5% B to 45% B in 60 min, 45% B to 95% B in 1 min, sustaining 95% B for 20 min, from 95% B to 5% B in 1 min, and finally re-equilibrating with 5% B for 8 min. Xcalibur 2.1 system (Thermo Fisher Scientific) was used to record peptide spectra over the mass range of m/z 350–1500, and MS/MS spectra in information dependent data acquisition over the mass range of m/z 150–2000. Repeatedly, MS spectra were recorded followed by two data dependent collision induced dissociation (CID) MS/MS spectra generated from two highest intensity precursor ions. Multiple charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics. MS/MS spectra were interpreted, and peak lists were generated by Proteome Discoverer 1.4.1.14 (Thermo Fisher Scientific). Searches were performed by using the SEQUEST HT (Thermo Fisher Scientific) against latest uniprot database for GABA_AR α 1 subunit (GBRA1_MOUSE, P62812), GABA_AR β 3 subunit (GBRB3_MOUSE, P63080) and GABA_AR γ 2 subunit (GBRG2_MOUSE, P22723). Searching parameters were set as follows: enzyme selected as used with three maximum missing cleavage sites, a mass tolerance of 10 ppm for peptide tolerance, 0.6 Da for MS/MS tolerance, dynamic of carbamidomethyl (C) and oxidation (M) and chemical modification (C, H, K, R, S, T, Y). Peptide identification and modification information returned from SEQUEST HT were manually inspected and filtered to obtain confirmed protein identification and modification lists of CID MS/MS.

Titration of quencher-ligand conjugates to labeled GABA_AR.

To examine fluorescent changes by the GABA-site quencher (**Gaba-Q**), HEK293T cells transfected with $\alpha 1/\beta 3/\gamma 2$ GABA_ARs were labeled with 2 μ M **CGAM-Gaba** for 4 h at 37 °C, then the cells were washed twice with serum free DMEM. The fluorescence intensity of the labeled GABA_ARs (F_0) was obtained by confocal microscopy. Subsequently, the labeled cells were treated with each concentration of **Gaba-Q**. After one-minute incubation, the fluorescence intensity of the labeled GABA_ARs upon the addition of quencher-ligand conjugate was measured (F_1). In all experiments, the imageJ software allowed subtraction of the background fluorescence and quantitative measurement of fluorescence intensity (F_0 and F_1) from plasma membrane in single cells.

To examine fluorescent changes by benzodiazepine-site quencher (**Bzp-Q**), the procedures were almost similar with that for **Gaba-Q** except for the incubation time with **CGAM-Bzp** (3 h).

The association constant ($K_{a(\text{quencher})}$, M^{-1}) of the quencher (**Gaba-Q** or **CGAM-Bzp**) for GABA_ARs was evaluated by fluorescence titration, in which the fluorescence intensity on cell surfaces was measured upon addition of the quenchers ($N=3$). $K_{a(\text{quencher})}$ (M^{-1}) was calculated by the Hill plot using the equation:

$$(1 - r) / r = K_{a(\text{quencher})} \times [\text{quencher}]$$

r: fluorescence intensity

Ligand binding assay using GABA_AR-based semisynthetic fluorescent biosensors

To construct fluorescent biosensor for GABA-site ligands, the HEK293T cells transfected with $\alpha 1/\beta 3/\gamma 2$, $\alpha 3/\beta 3/\gamma 2$, or $\alpha 5/\beta 3/\gamma 2$ GABA_ARs were labeled with 2 μ M **CGAM-Gaba** for 4 h at 37 °C, then the cells were washed twice with serum free DMEM. The fluorescence intensity of the labeled GABA_ARs (F_0) was obtained by confocal microscopy. Subsequently, the labeled cells were treated with 0.1 μ M **Gaba-Q**. After one-minute incubation with **Gaba-Q**, the fluorescence intensity of the labeled GABA_ARs upon the addition of quencher-ligand conjugate was measured (F_1). We termed these cells, HEK(Gaba). Then, the HEK(Gaba) was treated with various ligands. After one-minute incubation with each ligand, the fluorescence intensity of the labeled GABA_ARs was measured (F_2). In all experiments, the imageJ software allowed subtraction of the

background fluorescence and quantitative measurement of fluorescence intensity (F_1 and F_2) from plasma membrane in single cells.

To construct fluorescent biosensors for benzodiazepine-site ligands (HEK(Bzp)), the procedures were almost similar with that for GABA-site ligands except for the concentration of **Bzp-Q** (1 μ M).

The apparent affinity constant ($K_{app(ligand)}$, M^{-1}) of the each ligand for GABA_ARs was evaluated by fluorescence titration, in which the fluorescence intensity on cell surfaces was measured upon addition of the ligands (N=3). $K_{app(ligand)}$ (M^{-1}) was calculated by the Hill plot using the following equation:

$$r / (1 - r) = K_{app(ligand)} \times [ligand]$$

r: fluorescence intensity

The real association constant for the each ligand ($K_{a(ligand)}$) was calculated based on the following equation:

$$K_{a(ligand)} = K_{app(ligand)} + (K_{app(ligand)} \times K_{a(quencher)} \times [quencher])$$

Statistical analysis

All data are expressed as mean $\pm$ SD or SEM. Comparison of two groups was made by two-sided unpaired Student's *t*-test. Post hoc multiple comparisons were conducted by using one-way ANOVA and Dunnett's test. Difference with $P < 0.05$ was considered significant.

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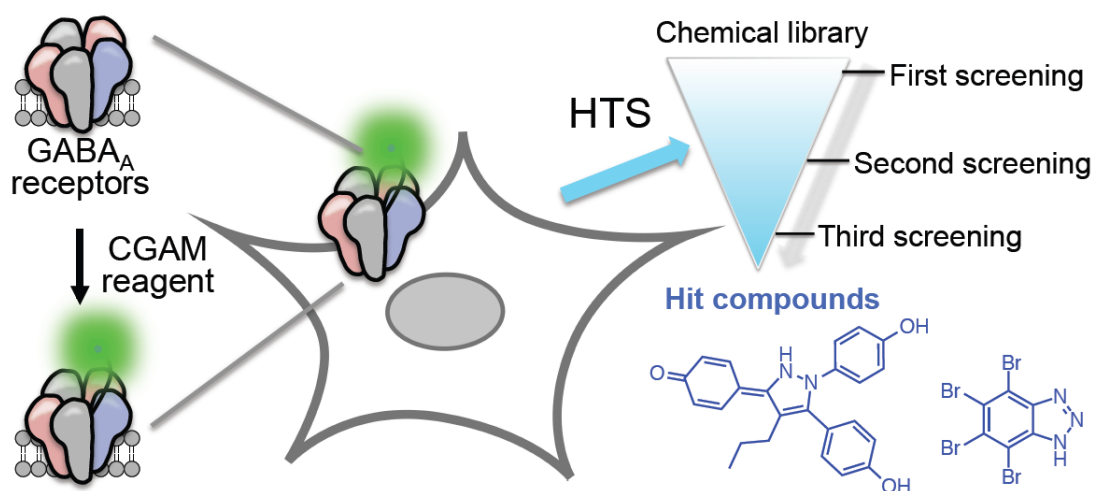
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Chapter 2

Discovery of new allosteric modulators for GABA_A receptor by ligand-directed chemistry

Abstract

Inhibitory neurotransmission in the brain is largely mediated by GABA_A receptors. Potentiation of GABA receptor activation through an allosteric benzodiazepine site by clinical used drug such as diazepam produces the sedative, anxiolytic, muscle relaxant, anticonvulsant and cognition-impairing effects. With the cell-based fluorescent biosensors for benzodiazepine-site ligand developed by ligand-directed chemistry in Chapter 1, I proceeded to screen a library of pharmacologically active compounds and sought to discover new allosteric modulators which interact with GABA_ARs. The screening followed by functional characterization revealed that one hit, 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), was a novel negative allosteric modulator capable of strongly suppressing the GABA-induced chloride currents. Thus, these semisynthetic biosensors represent versatile platforms for screening drugs to treat GABA_AR-related neurological disorders



2-1. Introduction

Benzodiazepine derivatives such as diazepam were introduced into clinical use in the 1960s due to its anxiolytic, anticonvulsant, sedative hypnotic, and muscle relaxant properties, and soon became the most commonly prescribed drugs in therapeutic use^{1,2}. Although the benzodiazepines were discovered by serendipity, their mode of action through the GABA_A receptor in brain was already well-established.

Fluorescent semisynthetic biosensors have attracted considerable interest in recent years as chemical tools for drug discovery, which can be applied to both biochemical- and cell-based high-throughput screening platforms³⁻⁹. Semisynthetic biosensors can be prepared using a site-specific chemical modification strategy with a synthetic fluorescent dye. For instance, Rauh and co-workers established a new biochemical screening system capable of identifying kinase¹⁰⁻¹², phosphatase¹³, and estrogen receptor inhibitors¹⁴. With regards to the construction of biosensors on live cells, Johnsson and co-workers recently designed a new class of semisynthetic biosensors termed SNAP-tag based indicator with a fluorescent intramolecular tether (Snifits) using an elaborate protein tag-based labeling technology coupled with Förster resonance energy transfer (FRET)¹⁵⁻¹⁷.

As described in Chapter 1, I have developed the semisynthetic biosensor for benzodiazepine-site ligands constructed by ligand-directed chemistry. Using this biosensor, I proceeded to screen a library of pharmacologically active compounds for identifying new allosteric modulators which interact with GABA_ARs. This biosensor is highly amenable to use in a high-throughput screening assay, and I discovered new small molecules acted as negative allosteric modulators for GABA_ARs.

2-2. Results and Discussions

2-2-1. Screening of small molecules bound to the benzodiazepine site on GABA_AR.

With the cell-based fluorescent turn-on biosensors for GABA_AR ligands in hand, I proceeded to screen a library of pharmacologically active compounds (LOPAC1280). Here, I used the OG-labeled GABA_AR biosensor for the benzodiazepine site. In this case, HEK293T cells expressing $\alpha 1/\beta 3/\gamma 2$ GABA_ARs were labeled with **CGAM-Bzp(1)**, and then the fluorescence signals were subsequently quenched by the addition of **Bzp-Q(2)** (**Fig. 1b**). This cell-based screening system was termed HEK(Bzp) and was designed as a biosensor for benzodiazepine-site ligands (**Fig. 1a**).

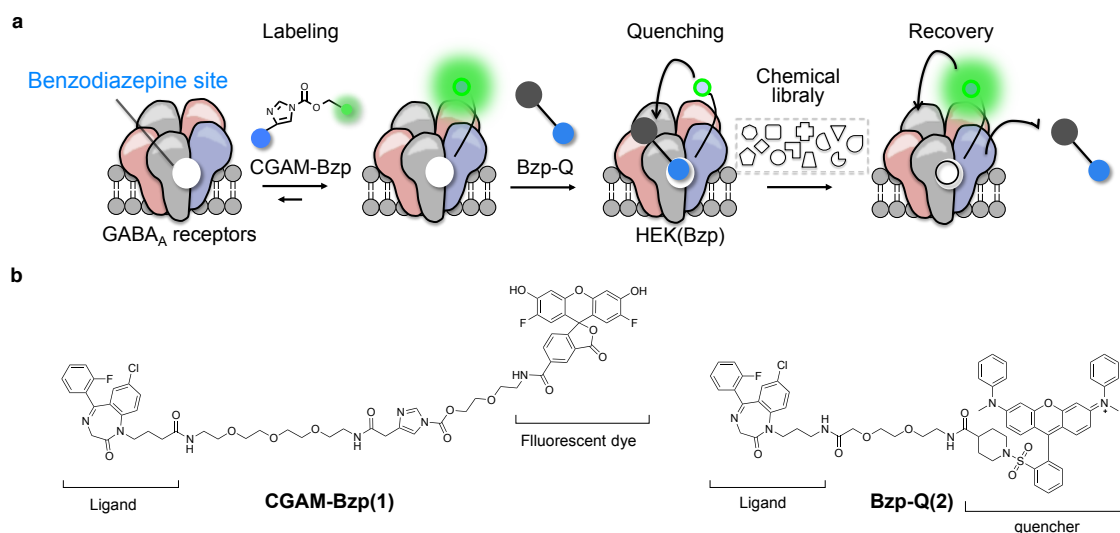


Fig. 1 Construction of GABA_AR-based semisynthetic fluorescent biosensors using CGAM-Bzp and Bzp-Q. (a) Schematic illustration for the ligand screening process using GABA_AR-based semisynthetic biosensors. (b) Chemical structures of **CGAM-Bzp** and **Bzp-Q** used in the present study.

Monitoring the fluorescence by confocal microscopy was conducted over three different steps to enhance the screening efficiency (**Fig. 2a**). For the first step, 24 or 32 individual compounds were mixed in the same tube, and the resulting mixtures were added to HEK(Bzp). As shown in **Figure 2b**, an increase in the fluorescence was observed in several groups, which provided four hit sample series (481–504, 577–600, 601–624, and 817–840) with a defined

threshold (F_2/F_1) of 1.2. In the second step, each hit group was divided into four sub-groups to give a total of 16 sub-groups containing six compounds. These compounds were then assayed, which resulted in the identification of four hit sample series (**499–504**, **583–588**, **601–606**, and **835–840**) (**Fig. 2c**). In the third screening step, each compound was individually added to HEK(Bzp), where any changes in the fluorescence were monitored. This process resulted in the identification of four hit compounds, including **501** (isoliquiritigenin, ILTG), **586** (4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol, PPT), **603** (flumazenil), and **838** (4,5,6,7-tetrabromo-2-azabenzimidazole, TBB) (**Fig. 2d, e**).

One of the hit compounds, flumazenil, which showed a high F_2/F_1 value ($F_2/F_1 = 1.6$), is a known ligand of the benzodiazepine site (*vide ante*). ILTG, which is a chalcone compound and guanylate cyclase activator, has been reported to exhibit a wide range of important pharmacological properties, including antioxidant, anti-inflammatory, anticancer, and anti-allergic activities. Furthermore, ILTG was recently reported as a positive allosteric modulator for GABA_ARs¹⁸. These hits clearly revealed the validity of my screening process. In contrast, there have been no reports pertaining to the affinity of PPT and TBB for GABA_ARs and these compounds are otherwise characterized as a selective inhibitor of casein kinase¹⁹ and an estrogen receptor α (ER α) agonist²⁰, respectively.

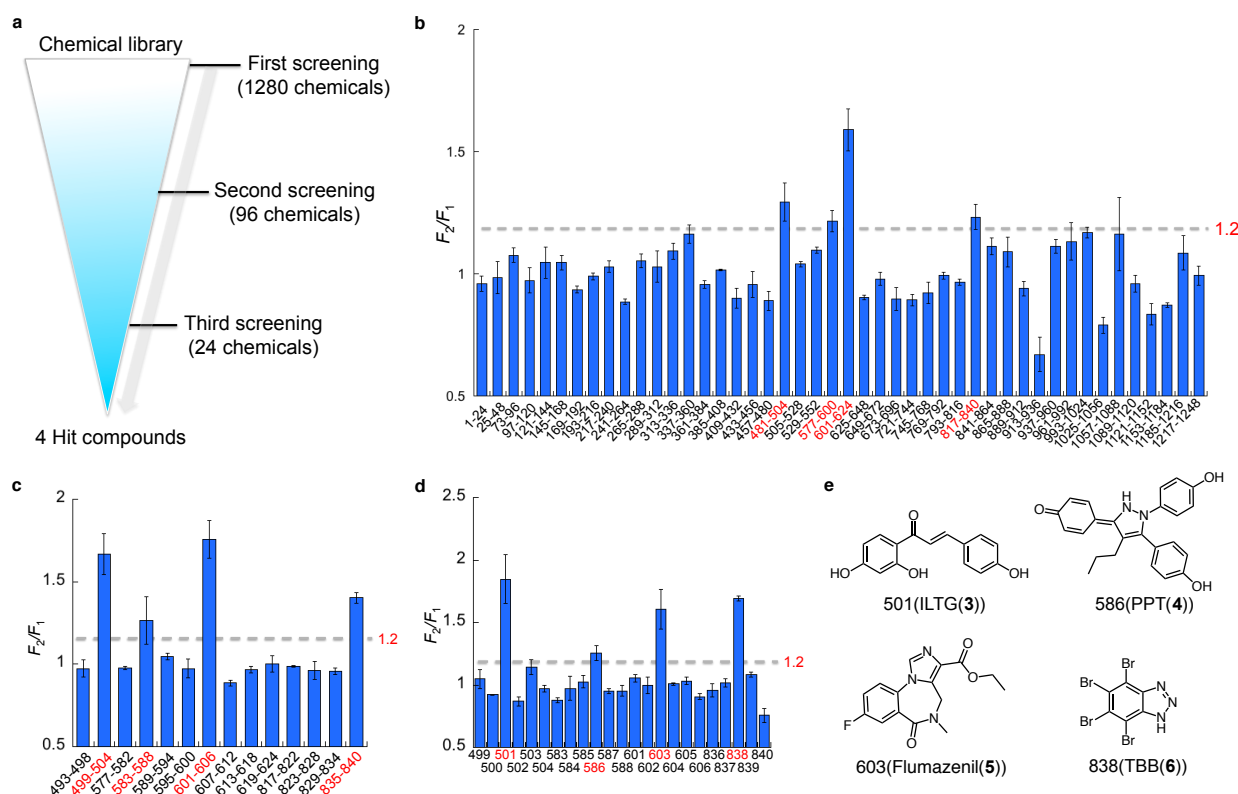


Fig. 2 High-throughput screening of small molecules bound to benzodiazepine site on GABA_AR using the semisynthetic biosensors, HEK(Bzp). (a) Flow chart of the screening process from a chemical library containing 1,280 compounds. (b) F_2/F_1 values in the first screening process. HEK(Bzp) was treated with samples including 24 or 32 chemicals. Final concentration of each chemical was 20 μ M. Threshold ($F_2/F_1 = 1.2$) in this screening is shown as a dashed line. $n = 3$. (c) F_2/F_1 values in the second screening process. Hit groups in the first screening was divided into 4 sub-groups, and HEK(Bzp) was treated with the sub-groups. Final concentration of each chemical was 20 μ M. $n = 3$. (d) F_2/F_1 values in the third screening process. In this process, HEK(Bzp) was treated with 50 μ M of each compound. (e) Chemical structure of hit compounds (ILTG, PPT, flumazenil, and TBB). $n = 3$. Data represent mean $\pm$ SEM.

2-2-2. Validation of hit compounds.

I next examined the binding property of these hit compounds in detail, by competitive radioligand binding assay, my BFQR-based fluorescent titration, and electrophysiological experiments. Among hit compounds, flumazenil and ILTG clearly inhibited radioactive ^3H -flumazenil binding to GABA_ARs, indicating that these competitively bind to the benzodiazepine sites (**Fig. 3a**). The K_i values were evaluated from the concentration-dependent curves ($K_i = 2.3 \text{ nM}$ and $1.4 \text{ }\mu\text{M}$ for flumazenil and ILTG, respectively), which are comparable to K_d values determined by my fluorescent methods ($K_d = 7.7 \text{ nM}$ and $0.63 \text{ }\mu\text{M}$ for flumazenil and ILTG, respectively) (**Fig 3b**). To my surprise, PPT and TBB did not inhibit ^3H -flumazenil binding, suggesting that these compounds do not competitively bind to the benzodiazepine site (**Fig. 3a**). In addition, on-cell competitive binding assay, where OG-conjugated flurazepam (**Bzp-OG(7)**) was used as a fluorescent indicator for detecting the competitive replacement with appropriate ligands, did not show any fluorescent changes by addition of PPT or TBB (**Fig. 3c-e**). In contrast, my BFQR-based fluorescent assay system clearly showed the typical saturation curves of the fluorescent (F_2/F_1) changes by addition of PPT or TBB by varying these concentration (**Fig. 3b**). Besides, the electrophysiological assay agreed well with the results of my fluorescent assay, showing that both PPT and TBB reversibly inhibited GABA-induced chloride currents with the concentration-dependent manner (**Fig. 3f,g**). The IC_{50} value for PPT or TBB was determined to be $7.3 \text{ }\mu\text{M}$ or $6.3 \text{ }\mu\text{M}$, respectively from the change in the peak amplitude of GABA-induced current (**Fig. 3g**). Given these results, it was considered that PPT and TBB do not competitively bind to the benzodiazepine site, rather bind to other (possibly adjacent) binding sites. Indeed, the binding affinities were directly determined from my fluorescent titration, giving the K_d values for PPT and TBB to be 7.7 and $14.5 \text{ }\mu\text{M}$, respectively (**Fig. 3b**), which are comparable to IC_{50} values determined by the electrophysiological measurements. The fluorescent changes by PPT and TBB in my system would reflect FRET efficiency changes between labeled-OG and **Bzp-Q**, which is induced by structural changes of GABA_ARs upon direct binding of these compounds to GABA_ARs (**Fig. 3h**).

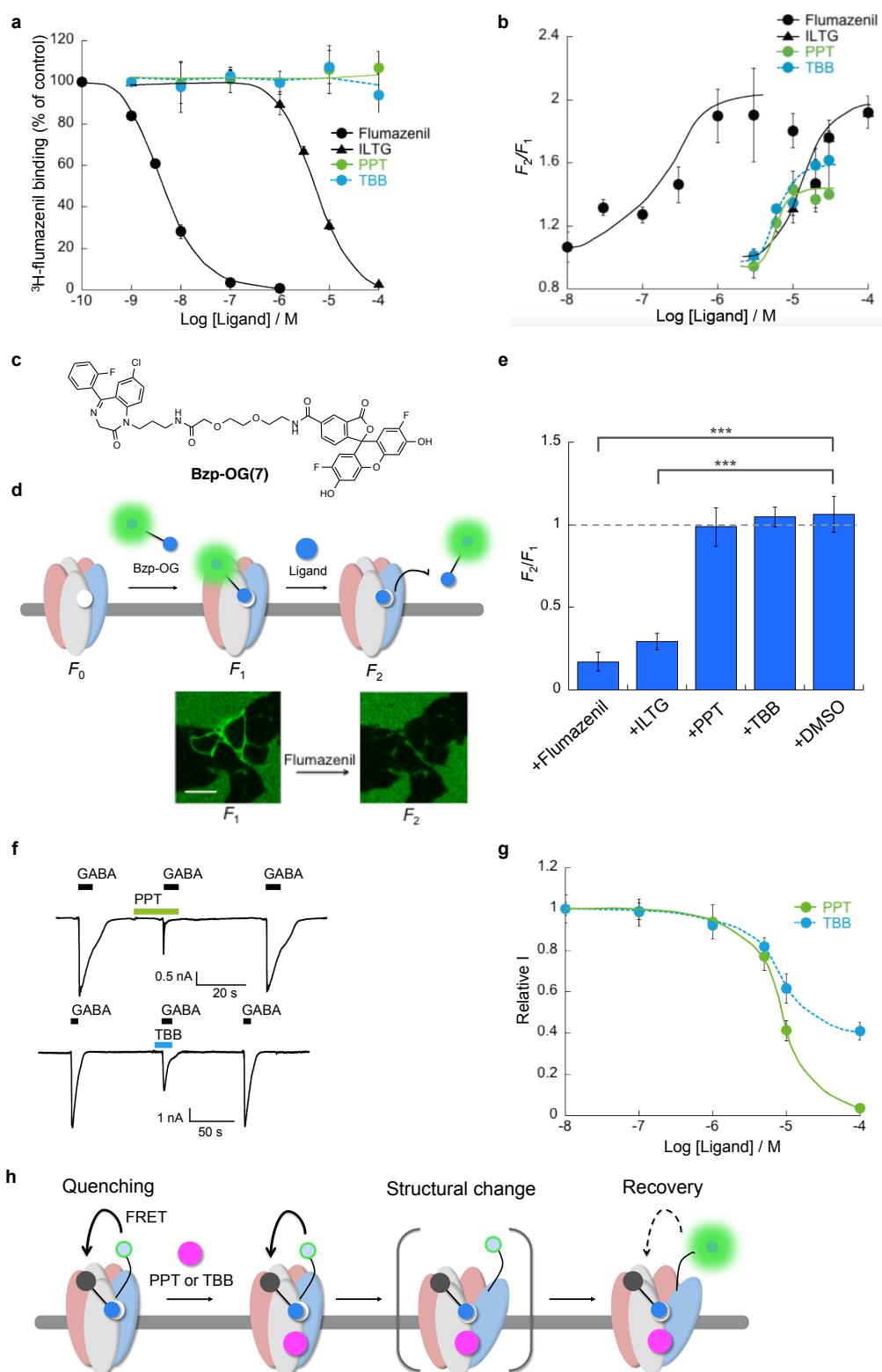


Fig. 3 Binding property of hit chemicals. (a) Concentration-dependency of hit compounds for the competitive radioligand binding assay using ^3H -flumazenil. $n = 3$. (b) Concentration-dependency of PPT and TBB for the F_2/F_1 ratio. HEK(Bzp) was treated with various concentration of PPT or TBB. $n = 3$. (c-e) On-cell binding of **Bzp-OG** to GABA_A Rs and subsequent evaluation of competitive inhibition of **Bzp-OG** by hit compounds (Flumazenil,

ILTG, PPT, or TBB). (c) Chemical structure of **Bzp-OG** (d) Schematic illustration of the fluorescent changes (upper) and representative confocal images (lower) are shown. Confocal images of HEK293T cells transfected with $\alpha 1/\beta 3/\gamma 2$ GABA_ARs were obtained after addition of 0.3 μ M Bzp-OG to the culture medium. The image shows that not only the specific binding of Bzp-OG to GABA_ARs (F_1) but also high background from free Bzp-OG in the medium were observed. Then, the cells were treated with hit compounds, and confocal images (F_2) were similarly obtained. Concentration of each hit compound is the following: flumazenil (10 μ M), ILTG (0.1 mM), PPT (0.1 mM), and TBB (0.1 mM). Scale bars, 20 μ m. (e) Fluorescent turn-off ratio (F_2/F_1) after addition of each hit compounds. The fluorescent intensity was obtained from confocal imaging as described in d. (f) Effects of PPT and TBB on GABA-induced whole-cell currents in $\alpha 1/\beta 3/\gamma 2$ GABA_AR-expressing HEK293T cells. Representative time courses of the GABA-induced currents recorded at -60 mV by the addition of PPT or TBB are shown. Black, green and blue bars indicate the periods of application of 100 μ M GABA, 10 μ M PPT and 100 μ M TBB, respectively. (g) Concentration-response curve for the effect of PPT and TBB on the peak amplitude of 100 μ M GABA-induced current at -60 mV. The IC_{50} for PPT or TBB was 7.3 or 6.3 μ M, respectively. $n = 5-14$. (h) The presumed binding mode of PPT or TBB to GABA_ARs.

2-2-3. Structure-activity relationship study of TBB derivatives.

Given that a range of TBB derivatives can be readily prepared, I conducted a basic structure-activity relationship study of this hit using HEK(Bzp). As shown in **Fig. 4**, neither *mono*-bromo benzotriazole (MBB) nor *di*-bromo benzotriazole (DBB) led to an increase in the F_2/F_1 value, which indicated that the *tetra*-bromo substituents on the benzotriazole scaffold were essential for binding to the GABA_AR. Interestingly, the *N*-alkylation of the 1-position of the triazole ring (1-TBBP) led to an increase in the F_2/F_1 value, while *N*-alkylation at the 2-position (2-TBBP) had no discernible impact on the value. These results therefore indicated that the introduction of an appropriate group at the 1-position but not at the 2-position could be used as a potential strategy to modulate the affinity or selectivity of TBB derivatives for the GABA_ARs.

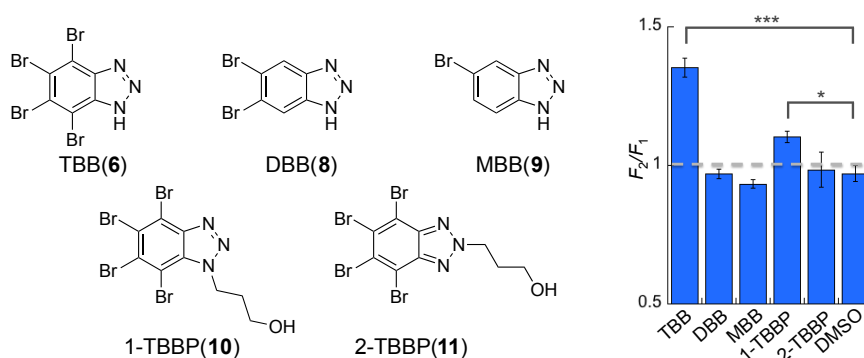


Fig. 4 Effect of TBB derivatives on the F_2/F_1 ratio. In the left, chemical structures of TBB derivatives are shown. In the right, the F_2/F_1 values for TBB derivatives are shown. The labeled cells were treated with 1 μ M **Bzp-Q**, which was followed by the addition of each compound at 10 μ M. $n = 3$. * $P < 0.05$; *** $P < 0.001$. Data represent mean $\pm$ SEM.

2-2-4. Mode of actions of PPT and TBB.

I next conducted a detailed investigation of the action of PPT toward the GABA_ARs using whole-cell-mode of patch-clamp recordings in HEK293T cells transfected with $\alpha 1/\beta 3/\gamma 2$ GABA_ARs. The addition of 100 μ M of PPT almost completely inhibited the GABA-induced current (**Fig. 3f,g**). The desensitization time constant (τ) of GABA_ARs was much smaller in the presence of PPT (**Fig. 5a**), implying that the inactivation properties of GABA_ARs were greatly facilitated by addition of PPT. Importantly, PPT did not have any effects on basal activity of GABA_AR currents (**Fig. 5b**). The concentration-dependent titration curve for GABA showed that the EC₅₀ value remained almost largely unchanged in the presence of 10 μ M of PPT (EC₅₀ = 20.2 or 16.1 μ M in the absence or presence of PPT, respectively) (**Fig. 5c**), which indicated that PPT was not competitive with GABA. These results therefore demonstrated that PPT was a negative allosteric modulator that elicits its action by weakening the efficacy of orthosteric agonist toward GABA_ARs. I also examined effects of TBB on GABA_AR function. Similar to PPT, application of TBB reversibly inhibited GABA-induced current without affecting the EC₅₀ value (**Fig. 3f** and **Fig. 5c**), and TBB did not have any effects on basal activity of GABA_AR currents (**Fig. 5b**). Unlike PPT, however, TBB did not change inactivation properties of GABA_ARs (**Fig. 5a**). Overall, both PPT and TBB were negative allosteric modulators that elicit its action by weakening the efficacy of orthosteric agonist toward GABA_ARs, although the effect on the channel kinetics is distinct among these compounds.

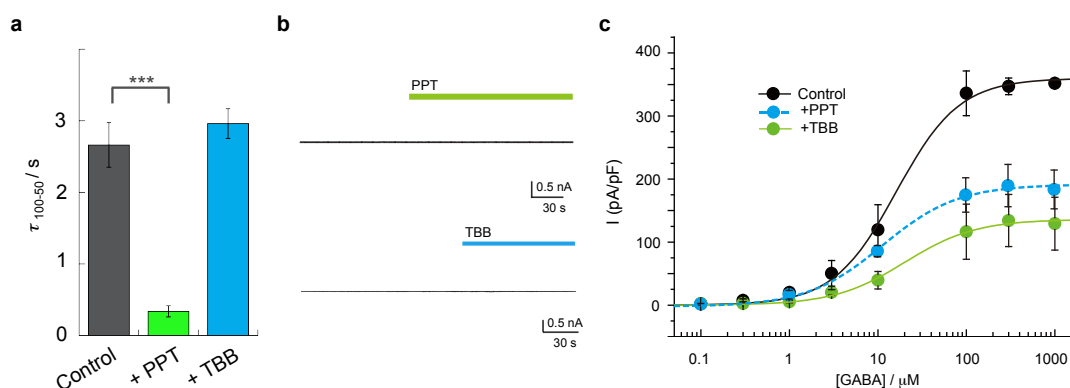


Fig. 5 Effects of PPT and TBB on GABA-induced whole-cell currents and the basal currents. (a) Time elapsed for 50% decay from the peak activation of GABA-induced current (τ_{100-50}) in the absence (control) and presence of 10 μ M PPT or 100 μ M TBB. $n = 10$. (b) Representative time courses of whole cell currents recorded at -60 mV in HEK293T cells transfected with $\alpha 1/\beta 3/\gamma 2$ GABA_AR by the addition of PPT or TBB. Green, blue bars indicates the period of 10 μ M PPT and 100 μ M TBB application, respectively. $n = 5$. (c) Concentration-response curves for peak GABA-induced current in the absence (control) and presence of 10 μ M PPT or 100 μ M TBB. EC_{50} value for control or PPT was 20.2 or 16.1 μ M, respectively ($n = 5$). *** $P < 0.001$. Data represent mean $\pm$ SEM.

2-3. Conclusion.

Thanks to the high-sensitivity of FRET system for structural changes of GABA_ARs, my BFQR system successfully identified not only competitive benzodiazepine-site ligands (flumazenil and ILTG) but also ligands (PPT and TBB) that modulate the benzodiazepine site through the high-throughput screening of a chemical library (**Table 1a**). It is generally accepted that competitive radioligand binding assay, a traditional method for examining drug-protein interactions, is difficult to apply for high-throughput screening. Recently, cell-based assay using FLIPRTM (fluorescence imaging plate reader) have been widely utilized in the high-throughput drug screening, but it gives us indirect information (e.g. intracellular Ca²⁺ concentration changes) monitored by fluorescence change using limited fluorescent indicators (e.g. Ca²⁺-sensitive dyes). Compared with them, my method allows for evaluating the direct binding to GABA_AR with the high-throughput manner, while the experimental protocols are almost comparable to other cell-based methods (**Table 1b**). There may be another approach as an on-cell competitive binding assay using fluorophore-ligand conjugates. However, the ligand binding was detected by the fluorescent decrease not by the increase, and the rather high background fluorescence was observed in the extracellular areas, both of which are sometimes problematic for evaluating precise ligand affinities. More importantly, I did not observe any fluorescence changes by addition of PPT or TBB in this simple replacement assay. These results implied that my method is able to find the more diverse hit compounds relative to the simple replacement assay, although the action mechanism of them should be carefully examined by combination with other methods. It is also noted as a limitation of my methods, that it is rather difficult to construct fluorescent biosensors for the low affinity ligands due to the non-specific labeling, and for a ligand-binding site that was deeply buried within receptor proteins.

a

	Flumazenil	ILTG	PPT	TBB
Ligand binding				
Radioligand binding assay	+	+	—	—
BFQR-based fluorescent assay	+	+	+	+
Functional analysis				
Electrophysiological method	Neutral antagonist	PAM	NAM	NAM

b

Method	FRIPR assay	Radioligand binding assay	On-cell competitive binding assay	BFQR-based fluorescent assay	Electrophysiological method
Procedure	1) Cell culture ↓ 2) Transfection of POI ↓ 3) Chemical probe (Ca ²⁺ , membrane potential, etc) ↓ 4) Chemical library	1) Cell culture ↓ 2) Transfection of POI ↓ 3) Cell lysis ↓ 4) Radioligand ↓ 5) Chemical library	1) Cell culture ↓ 2) Transfection of POI ↓ 3) Dye-conjugate ligand ↓ 4) Chemical library	1) Cell culture ↓ 2) Transfection of POI ↓ 3) Chemical labeling + Quencher-ligand conjugate ↓ 4) Chemical library	1) Cell culture ↓ 2) Transfection of POI ↓ 3) Seal formation ↓ 4) Chemical library
High throughput screening	Yes	Difficult	Yes	Yes	Difficult
Readout mode	Cellular function	Binding	Binding	Binding and Conformation change	Activity (ion current)
Competitive binding	—	Yes	Yes	Yes	Yes
Non-competitive binding	—	No	No	Yes	Yes
Data analysis	Fluorescence intensity or Imaging	Radioactivity or Scintillation count	Imaging	Imaging	Patch clamp

Table 1 (a) Summary of hit compounds characterized by radioligand binding assay, BFQR-based fluorescent assay, and electrophysiological method. PAM or NAM indicates positive allosteric modulator, or negative allosteric modulator, respectively. **(b)** The flowcharts for FRIPR assay, radioligand binding assay, on-cell competitive binding assay, BFQR-based fluorescent assay, and electrophysiological method in drug screening. POI indicates protein of interest.

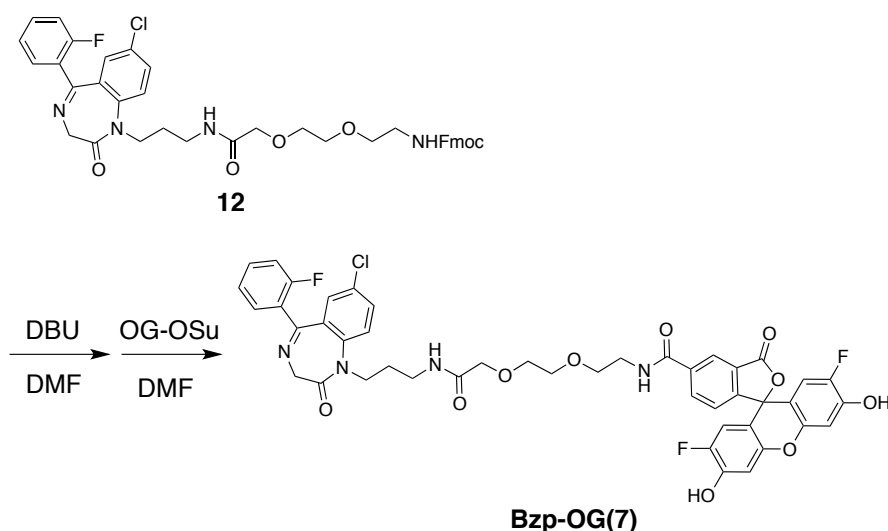
I have also demonstrated by the detailed electrophysiological measurements that PPT is capable of strongly suppressing the efficacy of orthosteric agonists of GABA_ARs. In general, benzodiazepine-site ligands act as allosteric modulators (positive, negative, or neutral mode), and most of the known negative allosteric modulators of this site work by lowering the affinity of GABA and partially suppressing the GABA-induced current¹. In contrast, it was evident that PPT almost completely inhibited the GABA-induced current, which was the salient feature of this compound. In some cases, the mode of the allosteric effect is reliant on the composition of the different subtypes of the GABA_ARs. For instance, methyl β -carboline-3-carboxylate (β -CCM) acts as a positive allosteric modulator toward the $\alpha 1/\beta 1/\gamma 1$ and $\alpha 2/\beta 1/\gamma 1$ GABA_ARs, but acts as a negative allosteric modulator toward most of the other compositions²¹. It would therefore be interesting to carefully evaluate the pharmacological modes of action of PPT in a future study.

2-4. Experimental Section.

General materials and methods for organic synthesis

All chemical reagents and solvents were purchased from commercial sources (Wako pure chemical, TCI chemical, Sigma-Aldrich, Sasaki Chemical) and were used without further purification. Thin layer chromatography (TLC) was performed on silica gel 60 F254 precoated aluminum sheets (Merck). Chromatographic purification was performed using flash column chromatography on silica gel 60 N (neutral, 40–50 μm , Kanto Chemical). ^1H -NMR spectra were recorded on 400 MHz Varian Mercury. ^{13}C -NMR spectra were recorded on 600 MHz JNM-ECA. Chemical shifts were referenced to residual solvent peaks or tetramethylsilane ($\delta = 0$ ppm). High resolution mass spectra were measured on an Exactive (Thermo Scientific) equipped with electron spray ionization (ESI). Reversed-phase HPLC (RP-HPLC) was carried out on a Hitachi Chromaster system equipped with a diode array, and an YMC-Pack Triat C18 or ODS-A column.

Synthesis of **Bzp-OG(7)**.



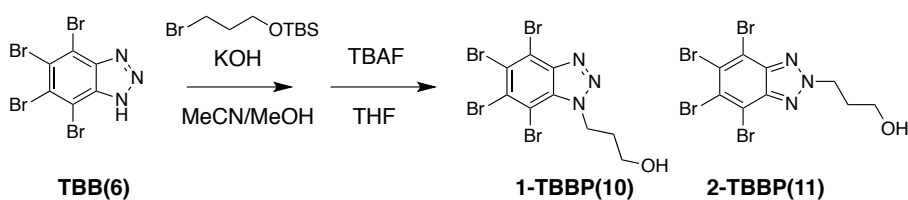
N-(2-(2-(2-((3-(7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-1-yl)propyl)amino)-2-oxoethoxy)ethoxy)ethyl)-2',7'-difluoro-3',6'-dihydroxy-3-oxo-3H-spiro[iso

benzofuran-1,9'-xanthene]-5-carboxamide (Bzp-OG(7))

To a stirred solution of compound **12** (1.0 mg, 1.4 μ mol) in dry DMF (200 μ l) was added DBU (20 μ l). The reaction mixture was allowed to stir for 1 h at room temperature. Then, OG-OSu (1.5 mg, 1.9 μ mol) was added to the solution. The reaction mixture was allowed to stir for 30 min at room temperature. The mixture was purified by HPLC (mobile phase; 0.1 % TFA CH₃CN (solvent A), 0.1 % TFA H₂O (solvent B)) to yield **Bzp-OG** (0.48 μ mol, 34 %) as a yellow solid.

¹H-NMR (600 MHz, CD₃OD): δ 8.48 (s, 1H), 8.19 (d, 1H, J = 7.8 Hz), 7.53-7.65 (m, 4H), 7.31 (t, 2H, J = 7.8 Hz), 7.12-7.16 (m, 2H), 6.83 (d, 2H, J = 7.2 Hz), 6.49 (s, 2H), 4.65 (d, 1H, J = 10.8 Hz), 4.41 (m, 1H), 3.97 (s, 2H), 3.83 (d, 1H, J = 10.2 Hz), 3.71-3.78 (m, 7H), 3.64 (t, 2H, J = 6.0 Hz), 1.72 (m, 1H), 1.59 (m, 1H). ¹³C-NMR-dept (150 MHz, CD₃OD): δ 134.20 (CH), 133.31 (CH), 132.54 (CH), 129.43 (CH), 125.95 (CH), 125.93 (CH), 125.74 (CH), 117.31 (CH), 117.17 (CH), 106.15 (CH), 72.11 (CH₂), 71.32 (CH₂), 71.29 (CH₂), 70.55 (CH₂), 57.74 (CH₂), 45.28 (CH₂), 41.13 (CH₂), 36.89 (CH₂), 28.85 (CH₂).

HR-ESI MS: calcd for C₄₅H₃₇ClF₃N₄O₁₀ [M+H]⁺ = 885.2150: obsd 885.2133.

Synthesis of 1-TBBP(10), 2-TBBP(11).

To a stirred solution of **TBB(6)** (27.0 mg, 62.0 μ mol) in dry MeCN (900 μ l) was added 1.7 % KOH MeOH solution (600 μ l), (3-bromopropoxy)(*tert*-butyl)dimethylsilane (628 mg, 2.48mmol). The reaction mixture was allowed to stir for 4 h at 60 °C. The solution was evaporated. The residue was purified by silica gel column chromatography (Hexane only $\rightarrow$ Hexane : CHCl₃ = 1:1 $\rightarrow$ Hexane : CHCl₃ : AcOEt = 5:5:1) to yield crude compound containing TBS-protected **1-TBBP**, TBS-protected **2-TBBP**. The residue was dissolved in THF (2.7 ml). Then, 1M TBAF in THF

solution (500 μ l) was added to the solution. The reaction mixture was allowed to stir for 1h at room temperature. The mixture was purified by HPLC (mobile phase; 0.1 % TFA CH_3CN (solvent A), 0.1 % TFA H_2O (solvent B)) to yield **1-TBBP(10)** (3.0 mg, 6.1 μ mol, 10 %) as a white solid, **2-TBBP(11)** (3.5 mg, 7.1 μ mol, 11 %) as a white solid. **1-TBBP**: ^1H -NMR (600 MHz, $\text{CDCl}_3:\text{CD}_3\text{CD}=\text{2} : 1$): δ 5.11 (t, 2H, $J = 11.4$ Hz), 3.69 (t, 2H, $J = 9.0$ Hz), 2.24 (q, 2H, $J = 9.0$ Hz). ^{13}C -NMR (150 MHz, $\text{CDCl}_3:\text{CD}_3\text{CD}=\text{2} : 1$): δ 145.81, 132.20, 129.77, 124.98, 116.62, 106.45, 57.98, 47.72, 33.47. HR-ESI MS: calcd for $\text{C}_9\text{H}_8\text{Br}_4\text{N}_3\text{O}$ $[\text{M}+\text{H}]^+ = 489.7401$: obsd 489.7400. **2-TBBP**: ^1H -NMR (600 MHz, $\text{CDCl}_3:\text{CD}_3\text{CD}=\text{2} : 1$): δ 4.90 (t, 2H, $J = 10.8$ Hz), 3.69 (t, 2H, $J = 10.2$ Hz), 2.36 (q, 2H, $J = 10.8$ Hz). ^{13}C -NMR (150 MHz, $\text{CDCl}_3:\text{CD}_3\text{CD}=\text{2} : 1$): δ 143.50, 126.64, 114.06, 58.79, 54.87, 32.97. HR-ESI MS: calcd for $\text{C}_9\text{H}_8\text{Br}_4\text{N}_3\text{O}$ $[\text{M}+\text{H}]^+ = 489.7401$: obsd 489.7399.

General methods for biological experiments

High-throughput screening of small molecules bound to benzodiazepine site on GABA_AR using the semisynthetic biosensors

Semisynthetic biosensors HEK(Bzp) was prepared as described in the section of ‘Ligand binding assay using GABA_AR-based semisynthetic fluorescent biosensors’. A library of pharmacologically active compounds (the LOPAC1280 (Sigma-Aldrich)) was utilized in the screening. To enhance the screening efficiency, ligand screening was divided into the following three steps.

In the first screening, 24 or 32 individual compounds (10 mM in DMSO) were mixed in the same tube, and evaporated to remove DMSO. Then, the residue was re-dissolved in 100 µl PBS containing 10% DMSO ([each compound] = 1 mM), and then centrifuged. The supernatant was added to HEK(Bzp) (final [each compound] = 20 µM). After one-minute incubation with each ligand, the fluorescence intensity of the labeled GABA_ARs was measured to obtain F_2/F_1 ratio. In this step, chemical mixtures showing fluorescence were excluded in the assay.

In the second screening, each hit group in the first screening was divided into 4 sub-groups. Two µl of 6 chemicals (10 mM in DMSO) was mixed in each sub-group ([each compound] = 1.7 mM). The mixture was added to HEK(Bzp) (final [each compound] = 20 µM), and F_2/F_1 ratio was obtained.

In the third screening, HEK(Bzp) was treated with each sample (final [each compound] = 50 µM), and F_2/F_1 ratio was obtained. In the all steps, threshold value of the F_2/F_1 ratio was defined as 1.2.

Radioligand binding studies

Membrane fraction from HEK293T cells transfected $\alpha 1/\beta 3/\gamma 2$ GABA_ARs were prepared as previously reported^{22,23}. Radioligand binding assay were performed with the membrane fractions including ³H-flumazenil (87 Ci/mmol, Perkin-Elmer) in a final volume of 0.1 ml containing 100 µg of protein in 50 mM Tris/citrate, pH7.4, 150 mM NaCl at 4 °C. For saturation analyses, each concentration of ³H-flumazenil was added. For competitive analyses, each concentration of

ligands (Flumazenil, ILTG, PPT, TBB, or **Bzp-Q**) was added in the presence of 1 nM ³H-flumazenil. After incubation of each sample for 1h at 4 °C, membranes were then filtered through Whatman GF/F filters, and the filters were rinsed twice with 5 ml of ice/cold 50 mM Tris/citrate buffer (pH7.4). Filters were transferred to scintillation vials and subjected to scintillation counting after addition of 4.0 ml of Clear-solI (Nacalai Tesque) scintillation fluid. The radioactivity was determined in a liquid scintillation counter (Packard). Nonspecific binding determined in the presence of 10 µM flumazenil was subtracted from total ³H-flumazenil binding to result in specific binding. Data points were fitted by pseudo-Hill plot for competitive experiments, and K_i values were calculated according to the Cheng-Prusoff equation²⁴.

Ligand binding assay for hit compounds (flumazenil, ILTG, PPT, and TBB)

The labeled cells by **CGAM-Bzp** were treated with 1 µM **Bzp-Q** (HEK(Bzp)). After one-minute incubation with **Bzp-Q**, the fluorescence intensity of the labeled GABA_ARs upon the addition of quencher-ligand conjugate was measured (F_1). After one-minute incubation with various concentration of hit compounds, the fluorescence intensity of the labeled GABA_ARs was measured (F_2). In all experiments, the imageJ software allowed subtraction of the background fluorescence and quantitative measurement of fluorescence intensity (F_1 and F_2) from plasma membrane in single cells.

For flumazenil and ILTG, other binding assay experiments indicated that these competitively bind to the benzodiazepine sites. Then, the K_d values were competitively calculated by the Hill plot using the following equation.

$$r / (1 - r) = K_{app(ligand)} \times [ligand]$$

r: fluorescence intensity

The real association constant for the each ligand ($K_{a(ligand)}$) was calculated based on the following equation:

$$K_{a(ligand)} = K_{app(ligand)} + (K_{app(ligand)} \times K_{a(quencher)} \times [quencher])$$

For PPT and TBB, other binding assay experiments indicated that these non-competitively bind to the benzodiazepine sites. Then, the K_d values were directly calculated by the Hill plot using the following equation.

$$(1 - r) / r = K_{a(\text{ligand})} \times [\text{ligand}]$$

r: fluorescence intensity

On-cell competitive binding assay by Bzp-OG

HEK293T cells (2.0×10^5 cells) were plated on 35-mm dishes (BD Falcon). After 18 h of plating, the cells were transfected with $\alpha 1/\beta 3/\gamma 2$ GABA_ARs as described in ‘cDNA expression in HEK293T cells’. mCherry-N1 (Clontech) was also co-transfected as a transfection marker. After 24 h of transfection, the cells were dissociated by pipetting and re-seeded on glass-bottom dishes pretreated with poly-L-lysine. After 24 h of the re-seeding, the medium was replaced with serum-free DMEM containing 0.3 μ M **Bzp-OG** and 1% DMSO. After 5 min, the fluorescence intensity from plasma membrane (F_1) was obtained by confocal microscopy. Subsequently, these cells were treated with hit compounds (flumazenil, ILTG, PPT, or TBB). After one-minute incubation, the fluorescence intensity from plasma membrane upon the addition of hit compound (F_2) was measured. The Fluoview software (Olympus) allowed quantitative measurement of fluorescence intensity from plasma membrane of single cells.

Electrophysiology

For electrophysiological measurements, coverslips with cells were placed in the experimental chamber. Membrane currents were recorded at room temperature (25 °C) in the whole-cell mode of patch clamp technique with an Axopatch 200B (Molecular devices) patch-clamp amplifier. Patch electrodes having a resistance of 2–5 M Ω (with internal solution) were fabricated from borosilicate glass capillaries. Current signals were filtered at 5 kHz with a 4-pole Bessel filter and digitized at 20 kHz. The pCLAMP (version 10.5.1.0; Molecular devices) software was used for command pulse control, data acquisition and analysis. For whole cell recording, series resistance was compensated (to 70–80%) to minimize voltage errors. The extracellular solution contained (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose and 10 HEPES (pH adjusted to 7.4 with NaOH, and osmolality adjusted to 300 mmol kg⁻¹ with D-mannitol). Intracellular solutions contained (in mM): 55 K gluconate, 50 KCl, 26 NaCl, 0.5 CaCl₂, 3 MgCl₂, 2 Na₂ATP, 5 EGTA, 5 HEPES and 5 creatine-phosphate (pH 7.2 adjusted with KOH, and osmolality adjusted to 290

mmol kg⁻¹ with D-mannitol). Relative current (*I*) in **Figure 3g** was defined by the following equation; relative $I = I_A/I_{Ctl}$, where I_{Ctl} and I_A are the peak amplitudes of 100 μ M GABA-induced whole cell currents observed before and after PPT or TBB application, respectively. PPT or TBB concentration-GABA current inhibition curves were fitted to the logistic equation: $I = (A_{max} - A_0) / [1 + (X / IC_{50})^n] + A_0$, where I is the normalized current amplitude, X is the antagonist concentration; n is Hill coefficient; IC_{50} is the concentration of antagonist that generates half-maximal inhibition. In **Figure 5d**, GABA concentration-response curves were fitted to the logistic equation: $I = (A_{max} - A_0) / [1 + (X / EC_{50})^n] + A_0$, where I is current amplitude, X is GABA concentration; n is a Hill coefficient; EC_{50} is the concentration of GABA that generates half-maximal amplitude. Data points in figures represent the means of n individual measurements from different cells.

Statistical analysis

All data are expressed as mean $\pm$ SD or SEM. Comparison of two groups was made by two-sided unpaired Student's *t*-test. Post hoc multiple comparisons were conducted by using one-way ANOVA and Dunnett's test. Difference with $P < 0.05$ was considered significant.

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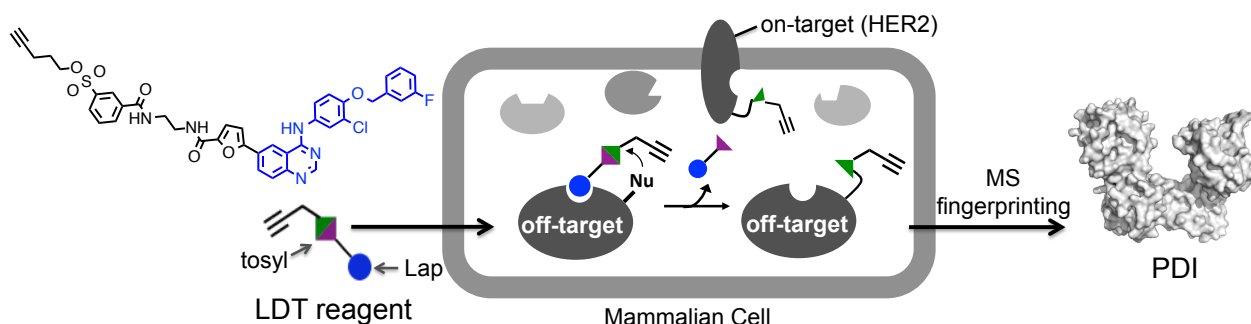
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Chapter 3

Live Cell Off-target Identification of Lapatinib Using Ligand-Directed Tosyl Chemistry

Abstract

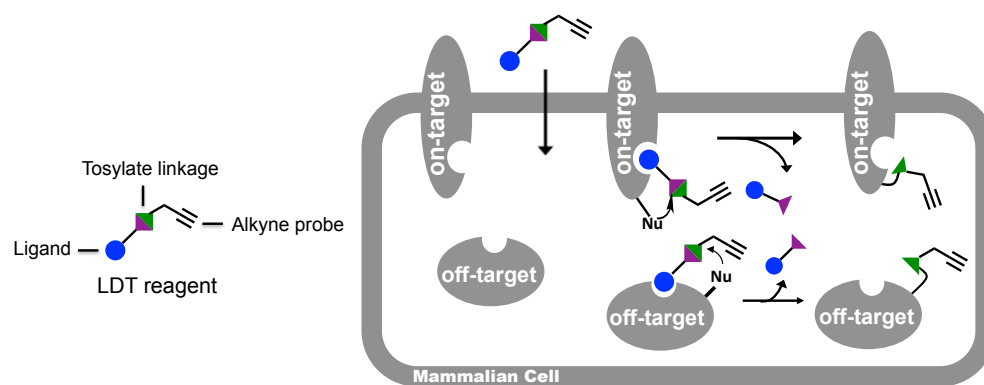
Drug discovery is a long and costly process, where one of the critical steps is identification of unintended off-targets. In vitro affinity-based assay using cell lysates, which were conventionally applied for off-target identification, has several drawbacks. Here I describe off-target identification under live cell condition, using ligand-directed tosyl (LDT) chemistry Hamachi group recently invented for covalent labeling of endogenous proteins. I focused on Lapatinib (Lap), a breast cancer drug which targets a membrane-bound receptor tyrosine kinase, HER2. Chemical labeling in live tumor cells revealed Lap-based LDT reagents bound to an off-target protein, as well as HER2. 2D-PAGE followed by MS fingerprinting analysis identified protein disulfide isomerase (PDI) as the off-target. Interestingly, I showed Lap regulates the PDI activity through the competitive binding with estradiol. LDT chemistry can be a useful tool for identifying off-targets of various drug candidates in live cells.



3-1. Introduction

One of the critical issues for drug discovery programs represents minimizing serious side effects for health, as well as maximizing the drug potency. In the molecular level, the side effect is caused by unexpected interactions between biomolecules other than targets (off-target) in many cases.^{1,2} Therefore, identification of such off-targets before clinical study should be greatly beneficial for anticipating potential side-effects and sidestepping them during drug development. The off-target identification has been mainly conducted by *in vitro* affinity-based assay using cell lysates to date.³⁻⁸ However, there are several drawbacks in the conventional methods, that is, (i) the cell lysate samples do not always reflect natural live cell environments where drugs operate on-target as well as off-targets, (ii) the non-covalent affinity based screening sometime excludes weak but specific interaction with off-targets. Recently, although some of the covalent trapping based methods were proposed,⁹⁻¹⁸ these are still insufficiently developed to date.

Hamachi group recently invented ligand-directed tosyl (LDT) chemistry for covalent labeling of endogenous proteins, which can be applied under live cell conditions.¹⁹⁻²¹ The proximity effects controlled by ligand-protein interactions efficiently facilitated a SN2 type of chemical reaction between an amino acid residue located around the ligand-binding pocket of proteins and the tosyl unit of LDT reagents. Because such proximity-driven covalent bond formation should be operated even for off-target proteins of the ligand, if any, as well as for on-target proteins, LDT chemistry was expected to be a useful tool for in-live cell identification of off-target proteins based on the covalent bond (**Scheme 1**).



Scheme 1 Schematic illustration of on-target and off-target protein labeling using LDT chemistry.

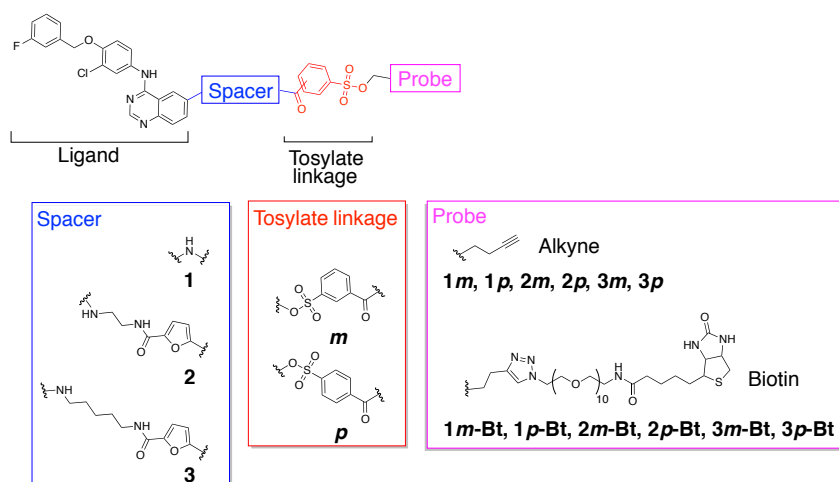
3-2. Results and Discussions

3-2-1. Molecular design of lapatinib-based LDT reagents on-target (HER2) labeling in test tube.

For the proof-of-principle experiments, I employed lapatinib (Lap),^{22,23} which is a U.S. Food and Drug Administration (FDA)-approved small molecule drug for breast cancer and other solid tumours. Lap inhibits a membrane-bound receptor tyrosine kinase, HER2 activity by competitive binding to the ATP binding site of its intracellular kinase domain. Based on the crystal structure of the HER2 kinase domain,^{24,25} I designed six different labeling reagents using Lap as a ligand part which have meta- or para-substituted tosyl unit and varied spacers between ligand and the SN2 reaction site as shown in **Fig. 1a**. As an appropriate probe with which proteins was modified, an alkyne tag was connected with the tosyl unit. These LDT reagents were prepared according to the synthetic protocols established in Hamachi group and new compounds were well characterized by NMR and high-resolution mass spectrometry.

Prior to live cell study, I initially confirmed the labeling efficiency of these reagents for on-target protein (HER2) in test tube. The biotin tethered labeling reagents (**1m-Bt**, **1p-Bt**, **2m-Bt**, **2p-Bt**, **3m-Bt**, **3p-Bt**), which were converted from the corresponding alkyne-type of LDT reagents (**1m**, **1p**, **2m**, **2p**, **3m**, **3p**) by click chemistry using biotin-N₃,^{26,27} were mixed with the water-soluble kinase domain of HER2 in aqueous buffer and then incubated at 25 °C for 10 h, followed by western blotting (WB) analysis. As shown in **Fig. 1b** and **1c**, the labeling efficiency is greatly dependent on the spacer length, that is, the shortest one (**1p-Bt**) is the most efficient (compare lane 2, 3, 5, 6, 8, 9 in **Fig. 1b**). Also, the clear inhibition of the labeling by addition of Lap (lane 4) revealed that the labeling was driven by the ligand-HER2 interaction.

a.



b.



c.

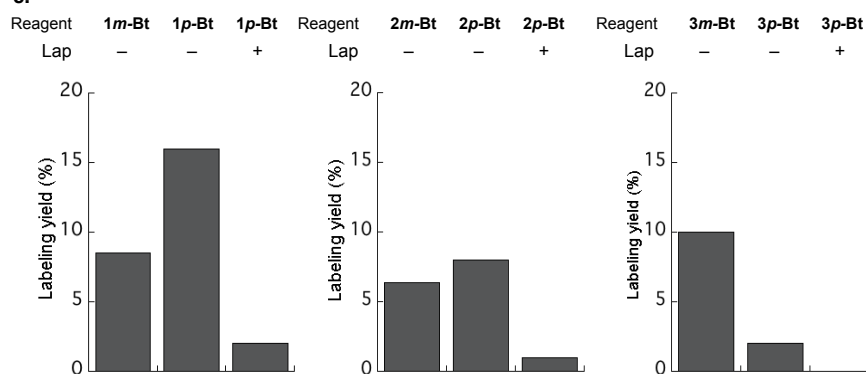


Fig. 1 Molecular structures of LDT reagents and HER2 labeling *in vitro*. **(a)** LDT reagent in this study. **(b)** *In vitro* HER2 kinase domain labeling. The reaction was carried out with 100 nM HER2 kinase domain and 1 μ M biotin tethered labeling reagents in the presence or absence of 20 μ M Lap in HEPES buffer (100 mM HEPES, 5 mM MgCl_2 , pH 7.5) at 25 $^{\circ}\text{C}$ for 10 h. Upper, biotin-blotting analysis using streptavidin (SAv)-HRP. Lower, western blotting analysis using rabbit anti-HER2 antibody. **(c)** Reaction yield of HER2 kinase domain labeling by biotin tethered labeling reagents. The labeling yields were determined by using biotin-labeled bovine albumin (Sigma) as a standard marker. The band intensity was obtained from western blotting as described in **(b)**.

3-2-2. On-target and off-target protein labeling using LDT reagents in live cells.

Having these labeling reagents in hand, I next conducted labeling experiments under live cell conditions using NCI-N87, a human gastric cancer cell line endogenously overexpressing HER2. After incubation with alkyne-type of labeling reagents (**1m**, **1p**, **2m**, **2p**) in the cell culture medium, lysis followed by click reaction to tether the biotin unit, the labeling reaction was evaluated by WB analysis from 250 kDa to 150 kDa (**Fig. 2a**). In good agreement with the labeling results in test tube, the clearest band for the labeled HER2 (ca 180 kDa) was observed by the labeling reagent **1p** having the shortest linker (lane 4), which strongly suggested the labeling occurred in the kinase domain of HER2. This band disappeared in the presence of inhibitor (Lap, lane 5), indicating the crucial role of the Lap-HER2 recognition for this labeling. We additionally confirmed the labeled protein as HER2, by immuno-precipitation (IP) using HER2 antibody (herceptin) which showed the stronger band by biotin blotting (**Fig. 2b**), implying that the LDT reagent **1p** is a powerful tool for selective labeling of intracellular kinase domain of HER2 in live cell conditions.

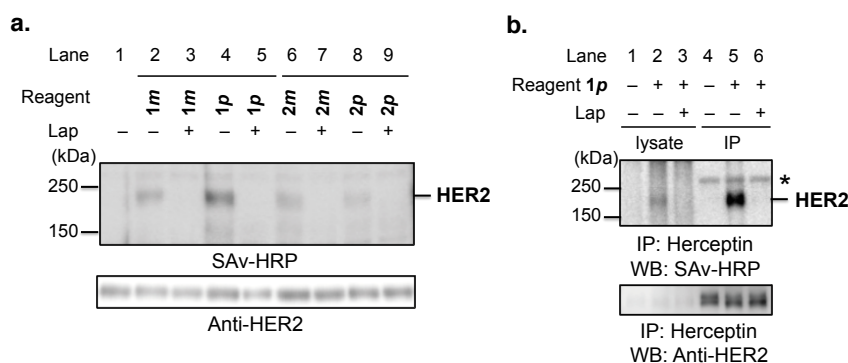


Fig. 2 Endogenous HER2 (on-target) labeling in live NCI-N87 cells **(a)** Chemical labeling of HER2 in live NCI-N87 cells by LDT reagent. NCI-N87 cells were treated with 5 μ M labeling reagent in DMEM (10% FBS) at 37 °C for 8 h with or without 20 μ M Lap. * indicates biotinylated proteins endogenously expressed in NCI-N87 cells. **(b)** Confirmation of chemical labeling of HER2 by immunoprecipitation (IP) by HER2 antibody. After labeling reaction using 1 μ M **1p** in the presence or absence of 20 μ M Lap followed by biotin modification, the cell lysate was immunoprecipitated with human anti-HER2 antibody, Herceptin. The immunoprecipitate was analyzed by biotin blotting with SAv-HRP (upper) or western blotting with rabbit anti-HER2 antibody (lower).

Interestingly, I noticed another band around 55 kDa appeared in the full WB analyses in the gastric and breast cancer cell lines (**Fig. 3a**). In gastric NCI-N87 cells, at the lower concentration of the labeling reagent (**1p**), the labeled HER2 band was predominantly detected, whereas the 55 kDa band slightly appeared in its intensity by the increment of **1p** (**Fig. 3b**). Like HER2 band, this band intensity decreased by addition of Lap, strongly implying that this labeling was also driven by the ligand-protein interaction (that can be an off-target for Lap). These data also demonstrated that an excess amount of the labeling reagent readily caused the off-target labeling in this case. As shown in Fig. 3a, this off-target band intensity was again varied with the labeling reagents, that is, **2m** produced the more intense band than **1m**, **1p** and **2p**. It may be note-worthy that the optimal LDT reagent for on-target labeling is distinct from that for off-targets, which would be attributed to the difference in the location and class of reactive amino acids between on-target and off-target proteins. We also carefully examined the intensity change of the labeling band for both HER2 and the off-target by varying the **2m** concentration (**Fig. 3c**), indicating that the off-target band intensity was stronger than that of HER2. The optimal structure for on-target and for off-target labeling is distinct each other in the spacer length and the orientation of tosyl unit. Furthermore, the similar off-target labeling occurred in a HER2-negative human breast cancer cell line, MCF7 (**Fig. 3d**), indicating that the off-target band is not HER2 fragment.

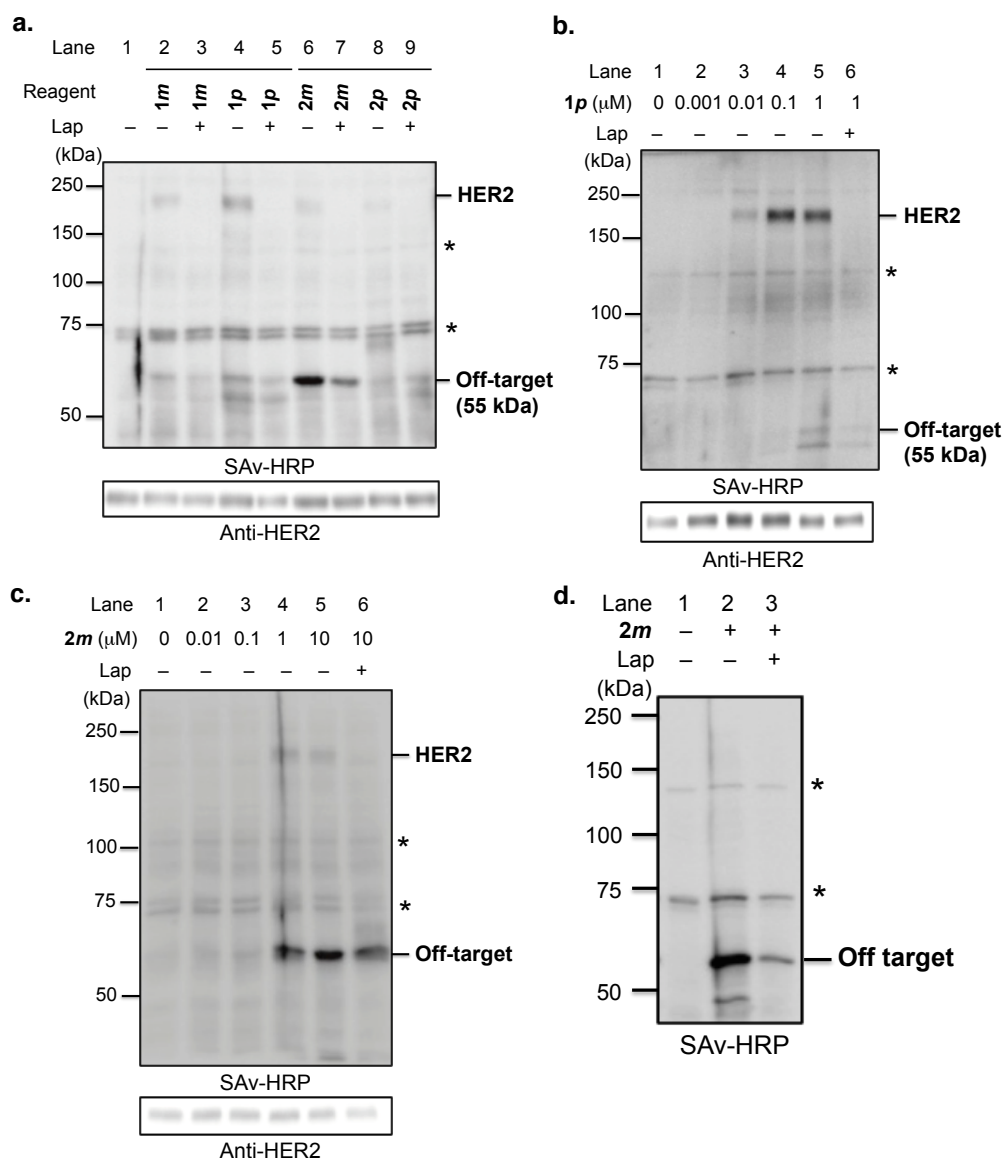


Fig. 3 Endogenous HER2 and off-target labeling in live NCI-N87 cells (a) Chemical labeling of HER2 in live NCI-N87 cells by LDT reagent. The blotting is full blot image of Fig 2a (b) Concentration-dependent labeling of HER2 by **1p**. Chemical labelings were conducted with **1p** under the same conditions as in (Fig. 2a). Upper, biotin-blotting analysis. Lower, western blotting analysis using anti-HER2 antibody. (c) Concentration-dependent chemical labeling of HER2 and an off-target protein by **2m**. NCI-N87 cells were treated with each concentration of **2m** in DMEM at 37 °C for 8 h with or without 30 μM Lap. Upper, biotin-blotting analysis. Lower, western blotting analysis using anti-HER2 antibody. (d) Chemical labeling of off-target in live MCF7 cells by **2m**. Chemical labelings were conducted with **2m** under the same conditions as in (c). * indicates biotinylated proteins endogenously expressed in MCF7 cells.

3-2-3. Off-target identification of lapatinib in NCI-N87 cells.

Subsequently, I sought to identify the off-target protein in NCI-N87 cells. After the labeling followed by click reaction, the biotinylated protein was roughly purified by anion-exchange chromatography. The resultant solution was subjected to 2D gel and the corresponding spot was detected by biotin blotting (**Fig. 4a**). The gel spot was excised, treated with in-gel trypsin digestion and then the resultant peptide fragments were determined by MS/MS analysis (TripleTOF 5600+, AB SCIEX). The MASCOT analysis of the obtained MS data (the protein score 7311 and the coverage 75%, **Fig. 4b**) revealed that the off-target protein was protein disulfide isomerase (PDIA1), an intracellular enzyme that catalyzes disulfide bond rearrangement from the mis-linked bond to the correct one for native protein folding.²⁸⁻³⁰ Reciprocal 2D-PAGE immunoblot analysis of the labeled proteins using anti-PDI antibody clearly showed that the off-target spot around 55 kDa merged well with that of PDI (**Fig. 4c**). The IP experiment using PDI antibody finally confirmed the off-target protein was PDI (**Fig. 4d**).

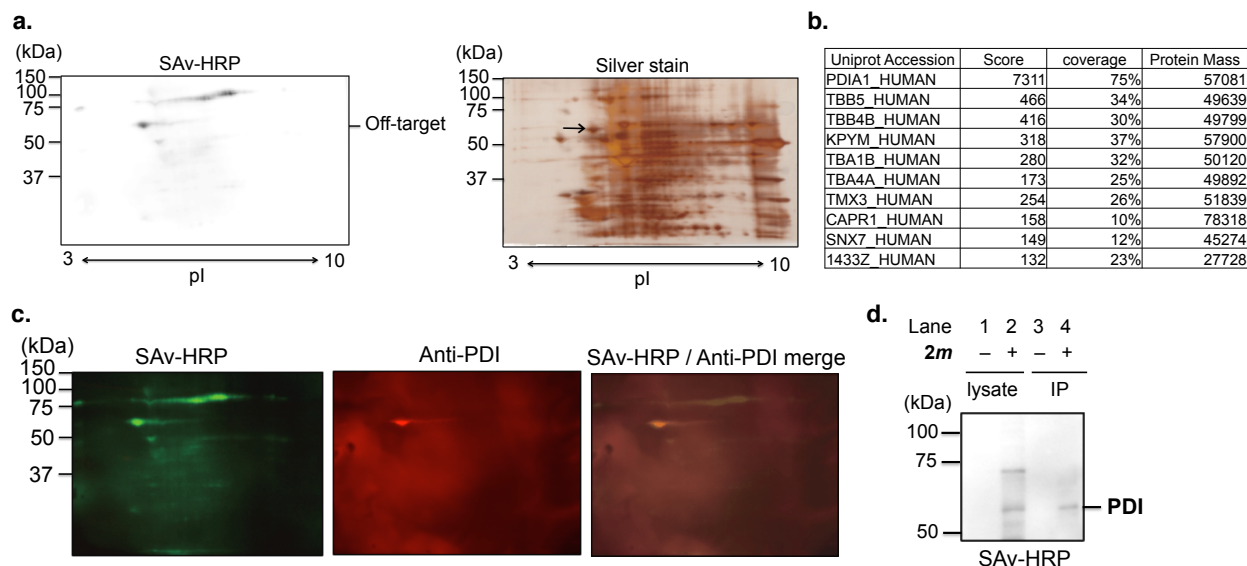


Fig. 4 Off-target identification of **2m** in live NCI-N87 cells (**a**) 2D-PAGE silver staining and western blotting of roughly purified cells lysate from NCI-N87 labeled with **2m**. Left, biotin-blotting analysis. Right, silver staining. (**b**) MS/MS analysis of off-target for Lap. The list of top 10 proteins by the MASCOT analysis are shown. (**c**) Reciprocal 2D-PAGE immunoblot analysis of labeled proteins by **2m** using anti-PDI antibody. The membrane was firstly stained with SAv-HRP for biotin-blotting analysis, then the membrane was reprobed with anti-PDI antibody for western blotting. Left, biotin-blotting analysis. Middle, western blotting analysis. Right, merged image. (**d**) Confirmation of chemical labeling of PDI in live NCI-N87 cells by immunoprecipitation by PDI antibody. After labeling reaction using 5 μ M **2m** followed by biotin modification, the cell lysate was immunoprecipitated with human anti-PDI antibody. The immunoprecipitate was analyzed by biotin blotting with SAv-HRP.

3-2-4. Identification of binding site of lapatinib.

In addition, the PDI labeling by a rhodamine tethered labeling reagent **2m-Rh**, which were converted from the corresponding alkyne-type of LDT reagents **2m** using rhodamine-N₃ in test tube was examined using purified PDI. The fluorescent gel image analysis showed that PDI was indeed labeled by **2m-Rh** in a dose-dependent manner (**Fig. 5a**). More importantly, the labeling was clearly inhibited by addition of 20 μ M of Lap (**Fig. 5b**) and the inhibition occurred in the concentration dependent manner (**Fig. 5c**).

It has been reported that PDI richly binds steroid hormones, such as estradiol (E2), mainly through hydrophobic interactions to regulate PDI activity.³¹⁻³⁶ In practice, we found that PDI labeling by **2m-Rh** was suppressed by the addition of E2 as well as Lap in vitro, suggesting that Lap binding to PDI may compete with E2 (**Fig. 5d**, lanes 2–4).

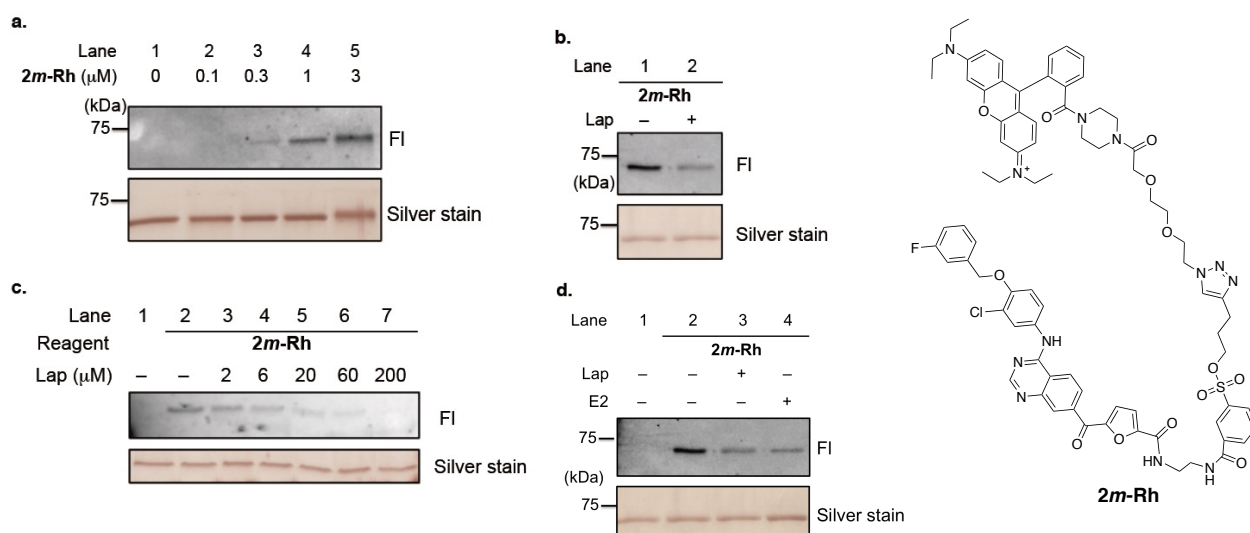


Fig. 5 Effect of Lap, E2, or ATP on PDI labelling by **2m-Rh**. **(a)** SDS-PAGE analysis of PDI labeling by in gel fluorescence imaging (FI) and silver staining. **(a-c)** Fluorescent (upper) and silver stained (lower) gel images of recombinant PDI labeled by **2m-Rh**. In **(a)**, the reaction was carried out with 220 nM PDI and each concentration of **2m-Rh** in HEPES buffer (50 mM HEPES, 3 mM DTT, pH 7.4) at 25 °C for 18 h. In **(b)**, the reaction was carried out in the presence or absence of 20 μ M Lap at 25 °C for 18 h. In **(c)**, the reaction was carried out in the absence or presence of each Lap at 25 °C for 18 h.

3-2-5. Effect of lapatinib on the off-target (PDI) activity.

In order to explore the biological relevance of Lap-PDI interaction, I next examined the impact of Lap (or E2) on the PDI activity by the conventional insulin turbidity assay (**Fig. 6a**).³⁴⁻³⁶ As previously reported,³¹⁻³³ 1 μ M of E2 decreased the initial rate for the insulin aggregation catalysed by PDI. Interestingly, the reduced PDI activity by E2 was gradually recovered by addition of Lap, although the simple Lap binding to PDI did not perturb the original PDI activity (**Fig. 6b**). This Lap-induced recovery of PDI activity showed the clear concentration dependence from 1 μ M to 10 μ M, indicating that the E2 bound to PDI was replaced with Lap. Such a finding may suggest an interesting possibility that Lap would regulate the PDI activity through competitive binding with E2 on PDI. In addition, it is proposed that PDI could serve as an intracellular reservoir for hormones including E2.³² which let us expect another possibility that the competitive binding of Lap releases E2 (estrogen receptor agonist) from PDI, resulting in the activation of hormonal receptors in cells (**Fig. 6c**).

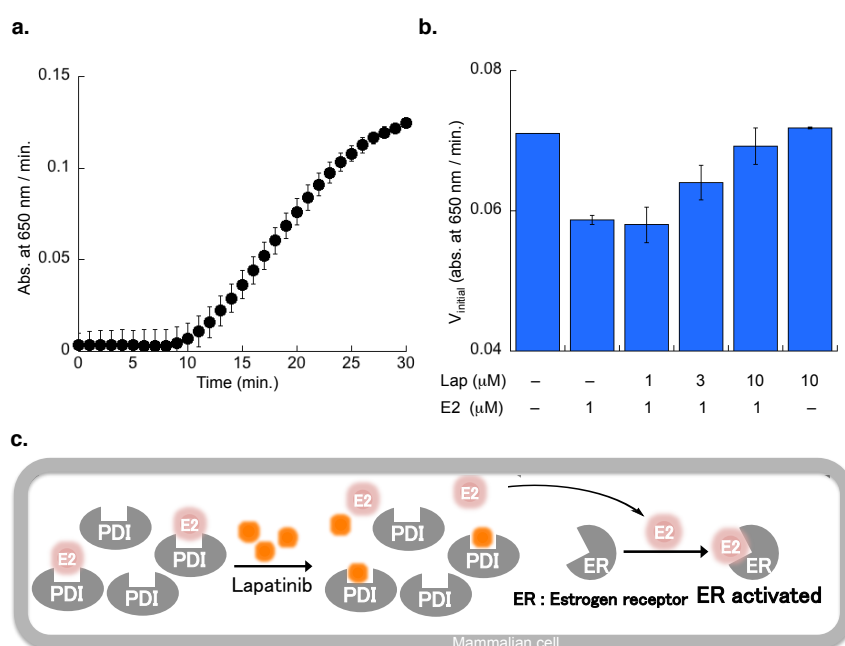


Fig. 6 Effect of Lap on PDI activity. **(a)** Time course of PDI reductase activity. Reductase activity was assessed by measuring the PDI-catalyzed reduction of insulin. In this assay, the aggregation of reduced insulin chains was measured using microplate reader at 650 nm. The experiments were performed in phosphate buffer (100 mM NaH_2PO_4 , 1 mM DTT, 2 mM EDTA, pH 7.0) containing 830 ng / ml PDI and 1.0 mg / ml bovine insulin at 25 °C. **(b)** Effect of Lap and E2 on the initial velocity of PDI reductase activity. Reductase activity was assessed by

measuring the PDI-catalyzed reduction of insulin. The experiments were conducted under the same conditions as in (a). (c) Presumed effect of lapatinib on the off-target (PDI) activity in cells.

3-3. Conclusion.

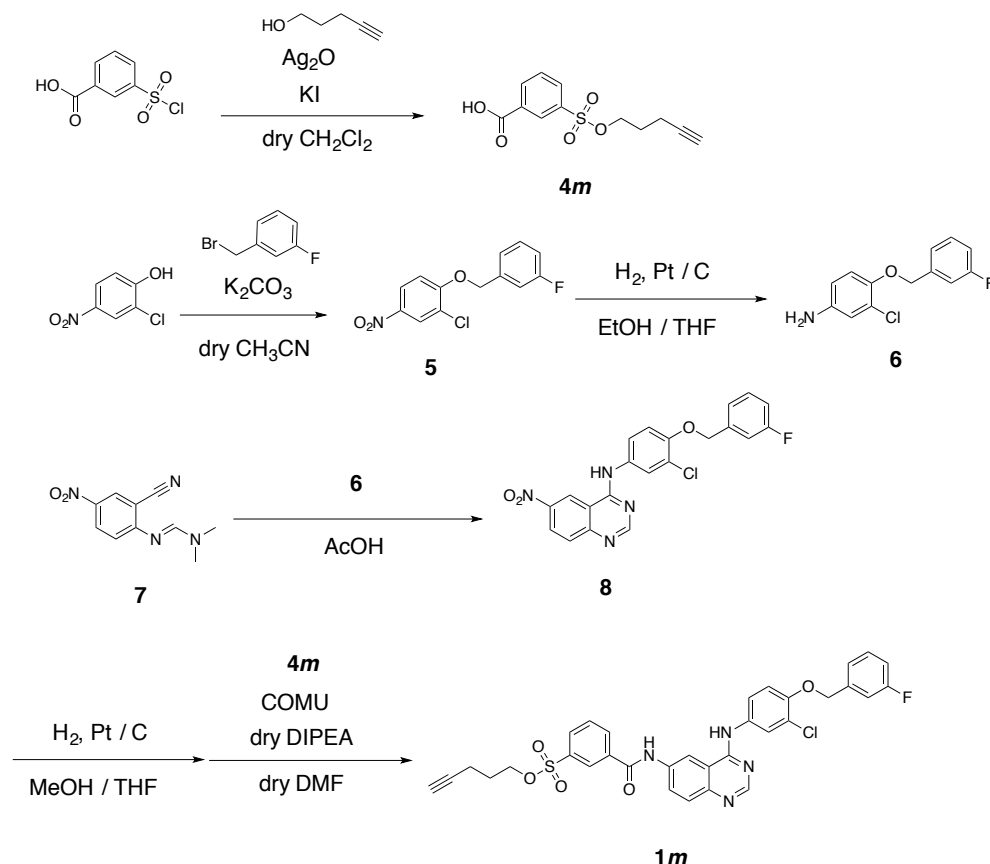
It is here demonstrated that LDT chemistry is applicable to detection of off-target proteins of a ligand in living cells, thanks to the covalent bond formation of endogenous proteins driven by the proximity effect. In this case, I firstly found PDI as a potential off-target of Lap, a FDA-approved anticancer drug. The binding affinity of Lap to PDI was estimated to be microM range, which highlighted an advantage of LDT labeling method, that is, relatively weak interaction partners can be picked up by covalent labeling under live cell conditions. On the basis of the subsequent activity assay, I also proposed a potential biological side-effect of Lap through the Lap/off-target (PDI) interaction. Recently, KINOME analysis revealed Lap binds not only to HER2 but also to many other proteins.^{37,38} However, screened proteins in this analysis are generally limited in kinase family only. In contrast, all cellular proteins are examined in my approach under live cell conditions. I believe that the present strategy shown here may be extended to other drugs or drug candidates, by which the more rapid and precise identification of their off-targets is facilitated.

3-4. Experimental Section.

Materials and Methods: All chemical and biochemical reagents were purchased from commercial sources (Wako pure chemical, TCI chemical, Sigma-Aldrich) and were used without further purification. All cell lines were obtained from ATCC. Thin layer chromatography (TLC) was performed on silica gel 60 F254 precoated aluminum sheets (Merck). Chromatographic purification was performed using flash column chromatography on silica gel 60 N (neutral, 40–50 μm , Kanto Chemical).

Physical Measurements: ^1H -NMR spectra were recorded on 400 MHz Varian Mercury spectrometer. Chemical shifts were referenced to residual solvent peaks or tetramethylsilane ($\delta = 0$ ppm). UV-vis absorption spectra were acquired on a Shimadzu UV-2550 spectrophotometer. High resolution mass spectra were measured on an Exactive (Thermo Scientific) equipped with electron spray ionization (ESI). Reversed-phase HPLC (RP-HPLC) was carried out on a Hitachi Chromaster system equipped with a diode array, and an YMC-Pack Triat C18 or ODS-A column. All runs used linear gradients of acetonitrile containing 0.1% TFA (solvent A) and 0.1% aqueous TFA (solvent B).

Biochemical experiments: SDS-PAGE and western blotting were performed using a Bio-Rad Mini-Protean III electrophoresis apparatus. Fluorescence and chemical luminescent signals were detected with Imagequant LAS 4000 (GE Healthcare).

Synthesis and Characterization:**Synthesis of 1m.****3-((pent-4-yn-1-yloxy)sulfonyl)benzoic acid (4m)**

To a stirred solution of 3-(chlorosulfonyl)benzoic acid (1.00 g, 4.53 mmol) in CH_2Cl_2 (45 ml) were added 4-Pentyn-1-ol (847 μl , 9.06 mmol), Potassium Iodide (KI) (150 mg, 0.90 mmol), Ag_2O (1.26 g, 5.44 mmol). The reaction mixture was allowed to stir for overnight at room temperature. The solution was filtered by Celite and purified by silica gel column chromatography (CHCl_3 : MeOH : AcOH = 20:2:1) and evaporated. To remove residual 3-(chlorosulfonyl)benzoic acid, the residue was dissolved in saturated aqueous NaHCO_3 . The aqueous layer was washed with CHCl_3 , acidified with 1N aqueous HCl , and extracted with CHCl_3 . The organic layer was dried over Na_2SO_4 , filter, evaporated to yield compound **4m** (62 mg, 0.23 mmol, 5 %) as a white powder. $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 8.64 (s, 1H), 8.38 (d, 1H, J = 8.0 Hz), 8.16 (d, 1H, J = 8.0 Hz), 7.71 (dd, 1H, J = 8.0, 8.0 Hz), 4.26 (t, 2H, J = 6.0 Hz), 2.28 (m, 2H), 1.87-1.93 (m, 3H).

2-chloro-1-((3-fluorobenzyl)oxy)-4-nitrobenzene (5)

To a stirred solution of 2-chloro-4-nitrophenol (9.37 g, 54.0 mmol), K₂CO₃ (18.66 g, 135 mmol) in dry CH₃CN (40 ml) were added *m*-fluorobenzyl bromide (8.50 g, 45.0 mmol) in dry CH₃CN (10 ml). The reaction mixture was allowed to stir for 18 h at 40 °C. The solution was evaporated and dissolved in AcOEt. The solution was washed with H₂O, saturated aqueous NaHCO₃, brine. The organic layer was dried over MgSO₄, filtered, evaporated to yield the compound **5** (12.6 g, 44.5 mmol, 99 %) as a pale yellow powder. ¹H-NMR (400 MHz, CDCl₃): δ 8.33 (d, 1H, *J* = 2.8 Hz), 8.14 (dd, 1H, *J* = 2.8, 9.2 Hz), 7.36-7.42 (m, 1H), 7.21-7.24 (m, 1H), 7.08-7.20 (m, 1H), 7.04-7.08 (m, 1H), 7.01 (d, 1H, *J* = 9.2 Hz), 5.26 (s, 2H).

3-chloro-4-((3-fluorobenzyl)oxy)aniline (6)

To a stirred solution of the compound **5** (6.00 g, 21.3 mmol) in THF : EtOH = 1 : 1 (35 ml) was added 5 % platinum-carbon (600 mg) at room temperature. The reaction mixture was allowed to stir for 3.5 h under hydrogen atmosphere. The solution was filtered by Celite and evaporated to yield the compound **6** (5.37 g, 21.3 mmol, quant.) as a brown solid. ¹H-NMR (400 MHz, CDCl₃): δ 7.30-7.36 (m, 1H), 7.17-7.22 (m, 2H), 6.97-7.02 (m, 1H), 6.79 (s, 1H), 6.76 (d, 1H, *J* = 2.8 Hz), 6.50 (dd, 1H, *J* = 2.8, 8.8 Hz), 5.03 (s, 2H).

***N*-(3-chloro-4-((3-fluorobenzyl)oxy)phenyl)-6-nitroquinazolin-4-amine (8)**

To a stirred solution of compound **7**³⁹ (236 mg, 1.08 mmol) in AcOH (2 ml) was added compound **6** (300 mg, 1.2 mmol) at room temperature. The reaction mixture was allowed to stir for 1h under reflux. The solid was filter and washed with Et₂O to yield compound **8** (227 mg, 0.53 mmol, 49 %) as yellow powder. ¹H-NMR (400 MHz, DMSO): δ 9.61 (s, 1H), 8.71 (s, 1H), 8.55 (dd, 1H, *J* = 2.8, 8.8 Hz), 8.00 (s, 1H), 7.92 (d, 1H, *J* = 8.8 Hz), 7.73 (dd, 1H, *J* = 2.8, 8.8 Hz), 7.46 (m, 1H), 7.30-7.34 (m, 3H), 7.18 (m, 1H), 5.26 (s, 2H).

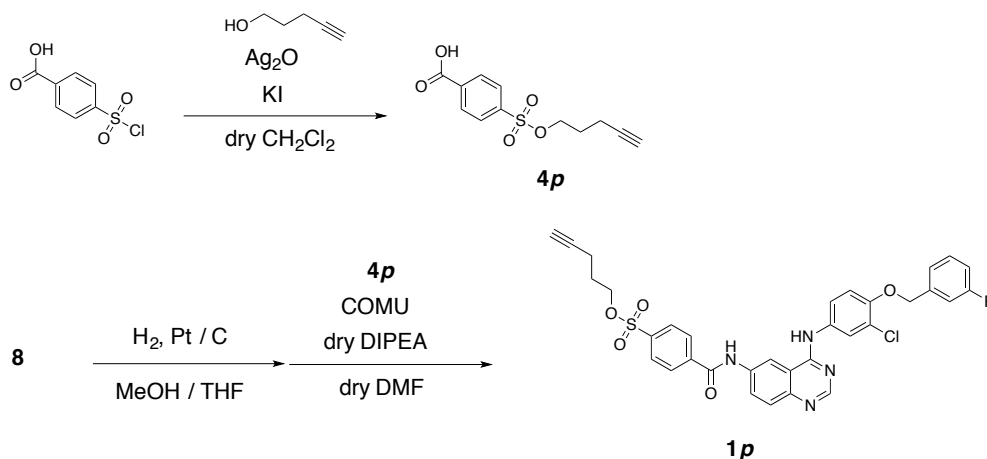
pent-4-yn-1-yl 3-((4-((3-chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)carbamoyl)benzenesulfonate (1m**)**

To a stirred solution of compound **8** (12 mg, 0.03 mmol) in THF : MeOH = 1 : 1 (1 ml) was added 5% *platinum-carbon* (1.5 mg) at room temperature. The reaction mixture was allowed to stir for 1 h under hydrogen atmosphere. The solution was filtered by Celite and evaporated. The residue was dissolved in dry DMF (0.3 ml). then, compound **4m** (8.5 mg, 0.03 mmol), (1-Cyano-2-ethoxy-2-oxoethylidenaminoxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) (17 mg, 0.05 mmol), dry N,N-diisopropylethylamine (DIPEA) (16 μ l, 0.09 mmol) were added to the solution. The reaction mixture was allowed to stir for 1 h at room temperature. The reaction mixture was diluted with CHCl_3 and washed with saturated aqueous NaHCO_3 and 5% citric acid. The organic layer was dried over Na_2SO_4 , filtered, evaporated. The residue was purified by silica gel column chromatography (CHCl_3 : MeOH = 40:1 $\rightarrow$ CHCl_3 : MeOH = 10:1) to yield Compound **1m** (2.5 mg, 3.9 μ mol, 13 %) as a brown solid.

$^1\text{H-NMR}$ (400 MHz, CD_3OD : CDCl_3 = 1 : 1): δ 9.05 (s, 1H), 8.65 (s, 1H), 8.55 (s, 1H), 8.37 (m, 1H), 8.29 (m, 1H), 8.12 (m, 1H), 7.77-7.86 (m, 3H), 7.11-7.37 (m, 5H), 7.01 (m, 1H), 5.21 (s, 2H) 4.25 (t, 2H, J = 5.6 Hz), 2.24 (m, 2H), 2.00 (s, 1H), 1.87 (m, 2H).

HR-ESI MS: calcd for $\text{C}_{33}\text{H}_{26}\text{ClF}_2\text{N}_4\text{O}_5\text{S}$ $[\text{M}+\text{H}]^+ = 645.1369$: obsd 645.1360.

Synthesis of 1p



4-((pent-4-yn-1-yloxy)sulfonyl)benzoic acid (4p)

To a stirred solution of 4-(chlorosulfonyl)benzoic acid (1.18 g, 5.34 mmol) in CH₂Cl₂ (54 ml) were added 4-Pentyn-1-ol (500 μ l, 5.34 mmol), KI (177 mg, 1.07 mmol), Ag₂O (1.5 g, 6.41 mmol). The reaction mixture was allowed to stir overnight at room temperature. The solution was filtered by Celite and purified by silica gel column chromatography (CHCl₃ : MeOH = 10:1) and evaporated. To remove residual 4-(chlorosulfonyl)benzoic acid, the residue was dissolved in saturated aqueous NaHCO₃. The aqueous layer was washed with CHCl₃, acidified with 1N aqueous HCl, and extracted with CHCl₃. The organic layer was dried over Na₂SO₄, filter, evaporated to yield compound **4p** (70 mg, 0.27 mmol, 5 %) as a white powder. ¹H-NMR (400 MHz, CDCl₃): δ 8.38 (d, 2H, J = 8.0 Hz), 8.16 (d, 2H, J = 8.0 Hz), 4.25 (t, 2H, J = 6.0 Hz), 2.28 (m, 2H), 1.87-1.93 (m, 3H).

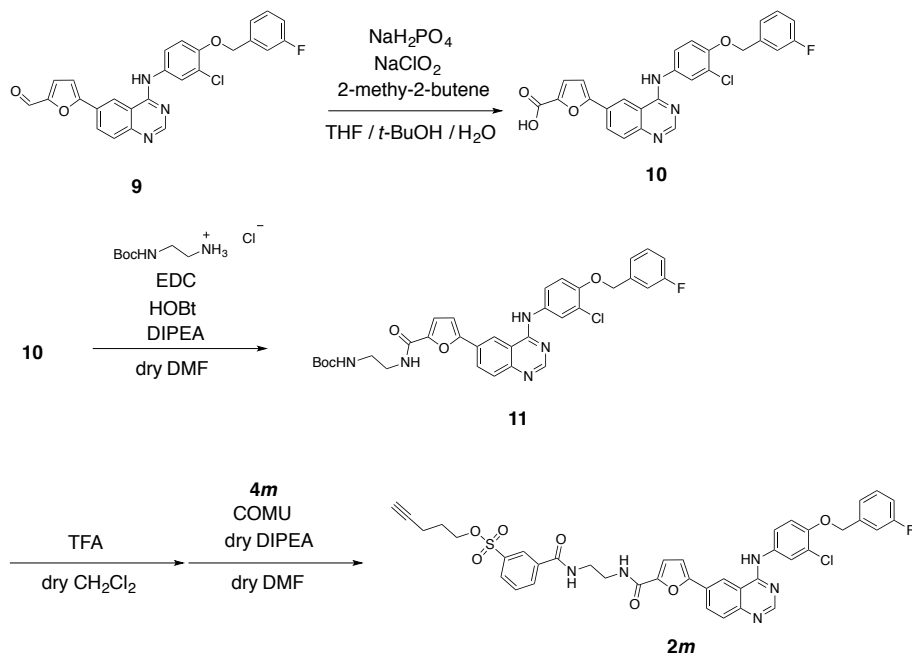
pent-4-yn-1-yl4-((4-((3-chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)carbamoyl)benzenesulfonate (1p)

To a stirred solution of compound **8** (12 mg, 0.03 mmol) in THF : MeOH = 1 : 1 (1 ml) was added 5% *platinum-carbon* (1.5 mg) at room temperature. The reaction mixture was allowed to stir for 1h under hydrogen atmosphere. The solution was filtered by Celite and evaporated. The residue was dissolved in dry DMF (0.3 ml). Then, compound **4p** (8.5 mg, 0.03 mmol), COMU (17 mg, 0.05 mmol), dry DIPEA (16 μ l, 0.09 mmol) were added to the solution. The reaction mixture was allowed to stir for 1 h at room temperature. The reaction mixture was diluted with CHCl₃ and washed with saturated aqueous NaHCO₃ and 5% citric acid. The organic layer was dried over Na₂SO₄, filtered, evaporated. The residue was purified by silica gel column chromatography (CHCl₃ : MeOH = 40:1 $\rightarrow$ CHCl₃ : MeOH = 10:1) to yield Compound **1p** (1.0 mg, 1.7 μ mol, 5 %) as a brown solid. ¹H-NMR (400 MHz, CD₃OD : CDCl₃ = 1 : 1): δ 9.09 (s, 1H), 8.67 (s, 1H), 8.22 (d, 2H, J = 8.4 Hz), 8.07 (d, 2H, J = 8.4 Hz), 7.82-7.87 (m, 2H), 8.12 (m, 1H), 7.77-7.86 (m, 3H), 7.55 (m, 1H), 7.11-7.37 (m, 5H), 7.02 (m, 1H), 5.23 (s, 2H), 4.24 (t, 2H,

$J = 5.6$ Hz), 2.26 (m, 2H), 2.04 (s, 1H), 1.87 (m, 2H).

HR-ESI MS: calcd for $C_{33}H_{26}ClFN_4O_5S$ $[M+H]^+ = 645.1369$; obsd 645.1361.

Synthesis of **2m**



5-((3-chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)furan-2-carboxylic acid (**10**)

To a stirred solution of compound **9** (250 mg, 0.528 mmol) in THF : t -BuOH : H_2O = 5 : 3 : 2 (10 ml) were added monosodium phosphate (320 mg, 2.67 mmol), 2-methyl-2-butene (370 mg, 5.28 mmol), sodium chlorite (181 mg, 1.60 mmol) at room temperature. The reaction mixture was allowed to stir for 1 h at room temperature. The reaction mixture was washed with brine and filtered. The residue was dissolved with THF and precipitated by hexane to yield compound **10** (255 mg, 0.521 mmol, 98 %) as an orange powder. 1H -NMR (400 MHz, CD_3OD): δ 8.51 (s, 1H), 8.27 (m, 1H), 7.89 (m, 1H), 7.84 (d, 1H, $J = 4.0$ Hz), 7.82 (d, 1H, $J = 4.0$ Hz), 7.61 (m, 1H), 7.38-7.44 (m, 1H), 7.25-7.28 (m, 2H), 7.19 (m, 1H), 7.03-7.08 (m, 2H), 7.19 (m, 1H), 5.24 (s, 2H).

***tert*-butyl(2-(5-(4-((3-chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)furan-2-carboxamido)ethyl)carbamate (11)**

To a stirred solution of compound **10** (100 mg, 0.2 mmol) in dry DMF (3 ml) were added *tert*-butyl (2-aminoethyl)carbamate hydrochloride (59 mg, 0.3 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (57 mg, 0.3 mmol), 1-hydroxybenzotriazole (HOBT) (46 mg, 0.3 mmol), DIPEA (50 μ l, 0.3 mmol). The reaction mixture was allowed to stir for 10 h at room temperature. The solution was purified by silica gel column chromatography (CHCl_3 : MeOH = 50:1) to yield compound **11** (89 mg, 0.14 mmol, 70 %) as a yellow solid. ^1H -NMR (400 MHz, CD_3OD): δ 8.77 (s, 1H), 8.67 (m, 1H), 8.28 (m, 1H), 7.93 (m, 1H), 7.73 (m, 1H), 7.60 (m, 1H), 7.36 (m, 1H), 7.21 (m, 2H), 7.18 (d, 1H, J = 4.0 Hz), 6.98-7.03 (m, 2H), 6.83 (d, 1H, J = 4.0 Hz), 5.17 (s, 2H), 3.49 (m, 2H), 3.33 (m, 2H), 1.23 (s, 9H).

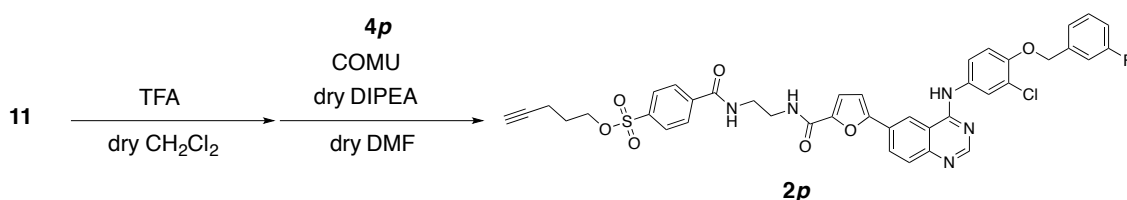
pent-4-yn-1-yl3-((2-(5-(4-((3-chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)furan-2-carboxamido)ethyl)carbamoyl)benzenesulfonate (2m)

To a stirred solution of compound **11** (32 mg, 0.05 mmol) in dry CH_2Cl_2 (1 ml) was added TFA (0.5 ml) at room temperature. The reaction mixture was allowed to stir for 1 h at room temperature. After azeotropic removal of TFA with toluene, the residue was dissolved in dry DMF (1.0 ml). Then, compound **4m** (13 mg, 0.05 mmol), COMU (28 mg, 0.08 mmol), dry DIPEA (8.7 μ l, 0.05 mmol) were added to the solution. The reaction mixture was allowed to stir for 1 h at room temperature. The solution was diluted with CHCl_3 and washed with saturated aqueous NaHCO_3 and 5% citric acid. The organic layer was dried over Na_2SO_4 , filtered, evaporated. The residue was purified by silica gel column chromatography (Hexane : AcOEt = 2:1 $\rightarrow$ CHCl_3 : MeOH = 10:1) and HPLC to yield compound **2m** (4 mg, 5.00 μ mol, 10 %) as a yellow solid. ^1H -NMR (400 MHz, CD_3OD : CDCl_3 = 1 : 1): δ 9.18 (s, 1H), 8.68 (s, 1H), 8.43 (m, 1H), 8.28 (s, 1H), 8.03 (m, 1H), 7.95 (m, 1H), 7.89 (m, 1H), 7.60-7.69 (m, 2H), 7.45 (m, 1H),

7.36 (m, 1H), 7.00-7.26 (m, 6H), 5.19 (s, 2H), 4.05 (t, 2H, $J = 6.0$ Hz), 3.78 (m, 2H), 3.65 (m, 2H), 2.12 (m, 2H), 1.95 (m, 1H), 1.74 (m, 2H).

HR-ESI MS: calcd for $C_{40}H_{33}ClFN_5O_7S$ $[M+H]^+ = 782.1846$: obsd 782.1840.

Synthesis of **2p**

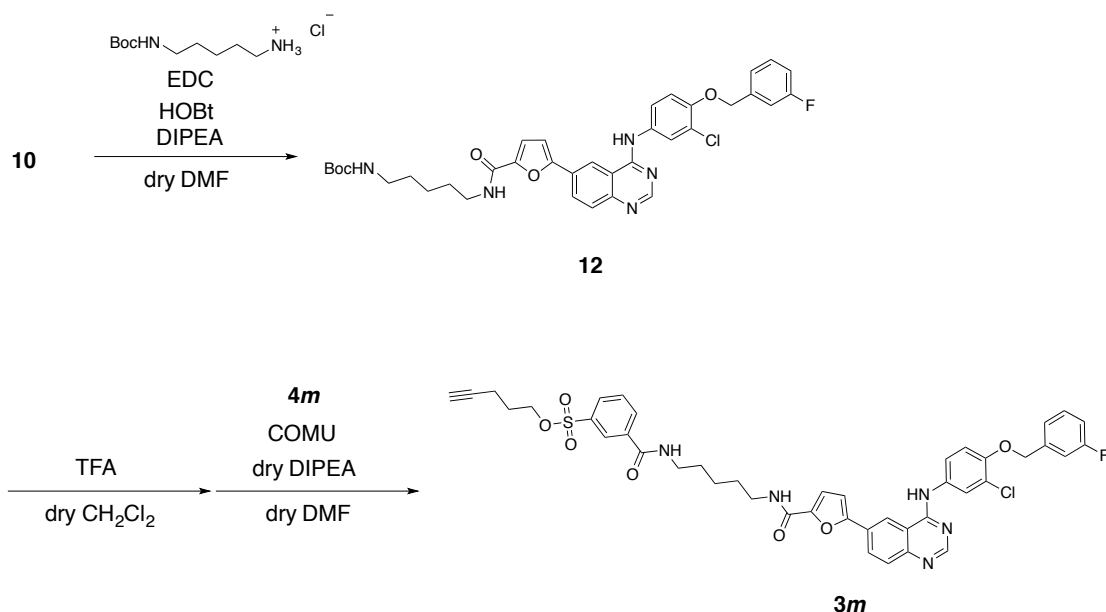


pent-4-yn-1-yl

4-((2-(5-(4-((3-chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)furan-2-carboxamido)ethyl)carbamoyl)benzenesulfonate (**2p**)

To a stirred solution of compound **11** (32 mg, 0.05 mmol) in dry CH_2Cl_2 (3 ml) was added *TFA* (1.5 ml) at room temperature. The reaction mixture was allowed to stir for 1 h at room temperature. After azeotropic removal of *TFA* with toluene, the residue was dissolved in dry DMF : dry $CH_2Cl_2 = 1:1$ (1.0 ml). Then, compound **4p** (13 mg, 0.05 mmol), COMU (28 mg, 0.08 mmol), dry DIPEA (8.7 μ l, 0.05 mmol) were added to the solution. The reaction mixture was allowed to stir for 1 h at room temperature. The solution was diluted with $CHCl_3$ and washed with saturated aqueous $NaHCO_3$ and 5% citric acid. The organic layer was dried over Na_2SO_4 , filtered, evaporated. The residue was purified by silica gel column chromatography (Hexane : AcOEt = 2:1 $\rightarrow$ $CHCl_3$: MeOH = 10:1) and HPLC to yield compound **2p** (1.5 mg, 2.00 μ mol, 4 %) as a yellow solid. 1H -NMR (400 MHz, $CDCl_3$): δ 9.18 (s, 1H), 8.51 (s, 1H), 8.33 (m, 2H), 7.82-7.97 (m, 5H), 7.46 (m, 1H), 7.34 (m, 1H), 7.00-7.24 (m, 5H), 6.85 (m, 1H), 5.11 (s, 2H), 4.15 (t, 2H, $J = 6.0$ Hz), 3.87 (m, 2H), 3.76 (m, 2H), 2.17-2.22 (m, 3H), 1.74 (m, 2H).

HR-ESI MS: calcd for $C_{40}H_{33}ClFN_5O_7S$ $[M+H]^+ = 782.1846$: obsd 782.1826.

Synthesis of **3m**

***tert*-butyl(5-(5-(4-((3-chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)furan-2-carboxamido)pentyl)carbamate (**12**)**

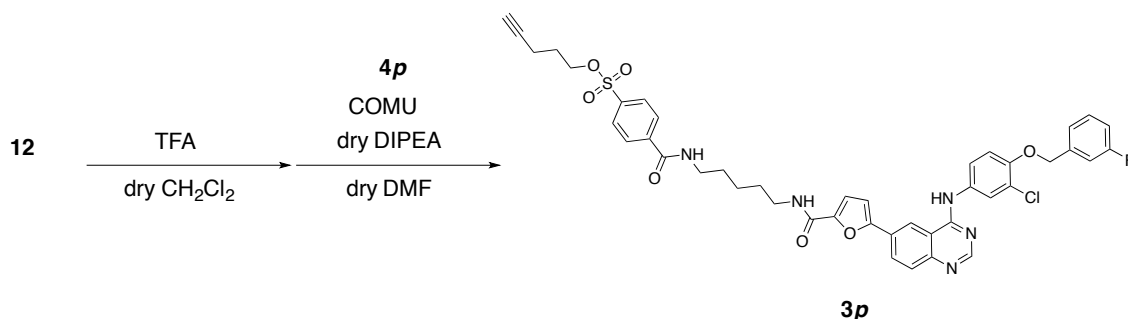
To a stirred solution of compound **10** (80 mg, 0.16 mmol) in dry DMF (2.3 ml) were added *tert*-butyl (5-aminopentyl)carbamate hydrochloride (69 mg, 0.25 mmol), EDC (48 mg, 0.25 mmol), HOBT (38 mg, 0.25 mmol), DIPEA (43 μl , 0.25 mmol). The reaction mixture was allowed to stir for 10 h at room temperature. The solution was purified by silica gel column chromatography (CHCl_3 : MeOH = 50:1) to yield compound **12** (55 mg, 0.08 mmol, 51 %) as a yellow solid. ^1H -NMR (400 MHz, CD_3OD): δ 8.55 (s, 1H), 8.40(s, 1H), 8.11 (m, 1H), 7.84 (m, 1H), 7.64 (m, 1H), 7.52 (m, 1H), 7.35 (m, 1H), 7.23 (m, 2H), 7.12 (d, 1H, $J = 4.0$ Hz), 6.98-7.04 (m, 2H), 6.94 (d, 1H, $J = 4.0$ Hz), 5.07 (s, 2H), 3.36 (m, 2H), 3.03 (m, 2H), 1.47-1.70 (m, 6H), 1.23 (s, 9H).

pent-4-yn-1-yl 3-((5-(5-(4-((3-chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)furan-2-carboxamido)pentyl)carbamoyl)benzenesulfonate (3m**)**

To a stirred solution of compound **12** (13 mg, 0.02 mmol) in dry CH_2Cl_2 (1 ml) was added TFA (0.5 ml) at room temperature. The reaction mixture was allowed to stir for 1 h at room

temperature. After removal of TFA with toluene, the residue was dissolved in dry DMF (0.4 ml). Then, compound **4m** (5.3 mg, 0.02 mmol), COMU (11 mg, 0.03 mmol), dry DIPEA (10 μ l, 0.06 mmol) were added to the solution. The reaction mixture was allowed to stir for 1.5 h at room temperature. The solution was diluted with CHCl_3 and washed with saturated aqueous NaHCO_3 and 5% citric acid. The organic layer was dried over Na_2SO_4 , filtered, evaporated. The residue was purified by silica gel column chromatography (CHCl_3 : MeOH = 40:1 $\rightarrow$ CHCl_3 : MeOH = 10:1) to yield compound **3m** (8.4 mg, 11.00 μ mol, 54 %) as a yellow solid. ^1H -NMR (400 MHz, CDCl_3): δ 8.80 (s, 1H), 8.62 (s, 1H), 8.07 (s, 1H), 7.97 (m, 1H), 7.83-7.85 (m, 3H), 7.60-7.64 (m, 2H), 7.33-7.40 (m, 2H), 6.97-7.25 (m, 6H), 5.11 (s, 2H), 4.03 (t, 2H, J = 6.0 Hz), 3.51 (m, 2H), 3.41 (m, 2H), 2.19 (s, 2H), 1.84 (m, 1H), 1.77 (m, 2H), 1.59-1.64 (m, 4H), 1.34-1.40 (m, 2H). HR-ESI MS: calcd for $\text{C}_{43}\text{H}_{39}\text{ClFN}_5\text{O}_7\text{S}$ $[\text{M}+\text{H}]^+ = 824.2316$: obsd 824.2299.

Synthesis of **3p**



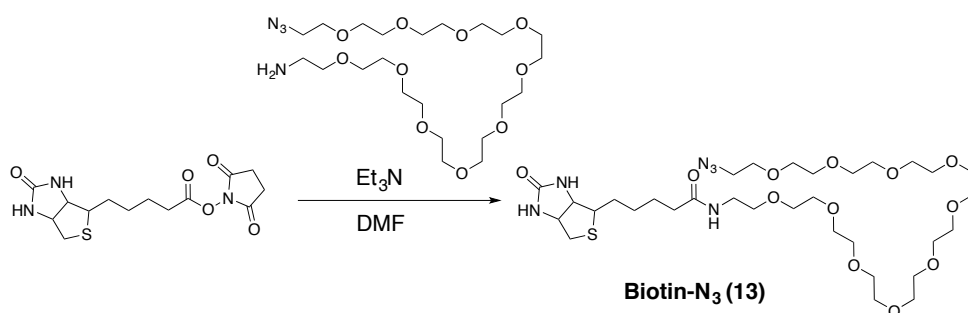
pent-4-yn-1-yl4-((5-(5-(4-((3-chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)furan-2-carboxamido)pentyl)carbamoyl)benzenesulfonate (**3p**)

To a stirred solution of compound **12** (34 mg, 0.05 mmol) in dry CH_2Cl_2 (1 ml) was added *TFA* (0.5 ml) at room temperature. The reaction mixture was allowed to stir for 2 h at room temperature. After azeotropic removal of TFA with toluene, the residue was dissolved in dry DMF (1.0 ml). Then, compound **4p** (13.0 mg, 0.05 mmol), COMU (28 mg, 0.08 mmol), dry DIPEA (8.7 μ l, 0.05 mmol) were added to the solution. The reaction mixture was allowed to stir for 1.5 h at room temperature. The solution was diluted with CHCl_3 and washed with saturated

aqueous NaHCO_3 and 5% citric acid. The organic layer was dried over Na_2SO_4 , filtered, evaporated. The residue was purified by silica gel column chromatography (Hexane : AcOEt = 2:1 $\rightarrow$ CHCl_3 : MeOH = 10:1) and HPLC to yield compound **3p** (1.0 mg, 1.20 μmol , 3 %) as a yellow solid. ^1H -NMR (400 MHz, CDCl_3): δ 8.92 (s, 1H), 8.57 (s, 1H), 8.41 (m, 1H), 8.29 (m, 1H), 7.84 (m, 2H), 7.72-7.76 (m, 3H), 7.30-7.43 (m, 2H), 6.93-7.19 (m, 6H), 5.15 (s, 2H), 4.07 (t, 2H, $J = 6.0$ Hz), 3.30-3.42 (m, 4H), 2.09-2.17 (m, 3H), 1.91 (m, 2H), 1.48-1.78 (m, 6H).

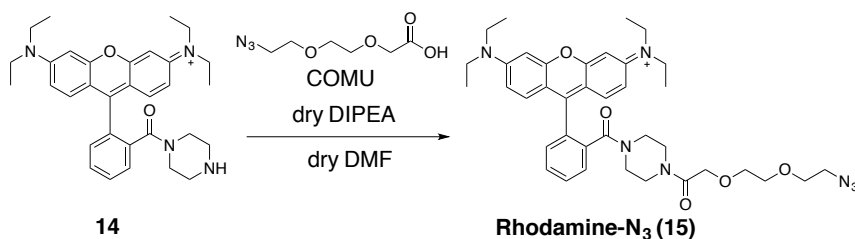
HR-ESI MS: calcd for $\text{C}_{43}\text{H}_{39}\text{ClFN}_5\text{O}_7\text{S}$ $[\text{M}+\text{H}]^+ = 824.2316$; obsd 824.2298.

Synthesis of Biotin- N_3 (**13**)



Biotin- N_3 (**13**)

To a stirred solution of 32-Azido-3,6,9,12,15,18,21,24,27,30-decaoxadotriacontan-1-amine (102.3 mg, 0.194 mmol) in dry DMF (1 ml) were added 2,5-dioxopyrrolidin-1-yl 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (65.3 mg, 0.191 mmol), Et_3N (30 μL , 0.216 mmol). The reaction mixture was allowed to stir for 2.5 h at room temperature. The reaction mixture was purified directly by HPLC to yield compound Biotin- N_3 (119.1 mg, 0.160 mmol, 83%) as a white gum. ^1H -NMR (400 MHz, CDCl_3): δ 6.94 (1H, t, $J = 5.0$ Hz), 6.32 (1H, s), 5.43 (1H, s), 4.47-4.53 (1H, m), 4.28-4.34 (1H, m), 3.61-3.69 (36H, m), 3.56 (2H, t, $J = 5.0$ Hz), 3.41-3.48 (2H, m), 3.39 (2H, t, $J = 5.0$ Hz), 3.11-3.18 (1H, m), 2.91 (1H, dd, $J = 12.8, 5.0$ Hz), 2.74 (1H, d, $J = 12.8$ Hz), 2.12-2.41 (4H, m), 1.62-1.81 (4H, m), 1.41-1.48 (2H, m).

Synthesis of Rhodamine-N₃ (15)**Rhodamine-N₃ (15)**

To a stirred solution of compound **14**⁴⁰ (20 mg, 0.04 mmol) in dry DMF (0.8 ml) were added COMU (23 mg, 0.06 mmol), dry DIPEA (20 μ l, 0.12 mmol) and 2-(2-(2-azidoethoxy)ethoxy)acetic acid⁴¹ (7.5 mg, 0.04 mmol, 1.0 eq). The reaction mixture was allowed to stir for 1 h at room temperature. The solution was diluted with CHCl₃ and washed with saturated aqueous NaHCO₃ and 5% citric acid. The organic layer was dried over Na₂SO₄, filtered, evaporated. The residue was purified by silica gel column chromatography (1% NH₃ aq. CHCl₃ : MeOH = 40:1 $\rightarrow$ 1% NH₃ aq. CHCl₃ : MeOH = 10:1) to yield Rhodamine-N₃ (27 mg, 0.04 mmol, 98 %) as a purple solid. ¹H-NMR (400 MHz, CDCl₃): δ 7.68-7.78 (m, 3H), 7.51 (m, 1H), 7.27 (d, 2H, J = 9.2 Hz), 7.07 (s, 2H, J = 9.2 Hz), 6.95 (s, 2H), 4.2 (s, 2H), 3.62-3.71 (m, 16H), 3.39 (m, 8H), 3.30-3.33 (m, 6H), 1.30 (t, 12H, J = 6.8 Hz).

Chemical labeling of recombinant HER2 kinase domain in test tube:

Biotin tethered labeling reagents (**1m-Bt**, **1p-Bt**, **2m-Bt**, **2p-Bt**, **3m-Bt**, **3p-Bt**) were prepared from alkyne labeling reagents (**1m**, **1p**, **2m**, **2p**, **3m**, **3p**) by click chemistry in test tube. Click chemistry condition were as follows: to a solution of alkyne labeling reagents (1 μ l, 5 mM in DMSO) was added Cu(MeCN)₄BF₄ (1 μ l, 75 mM in DMSO), bathophenanthroline disulfonic acid (BPAA) (1 μ l, 7.5 mM in DMSO : H₂O = 1:1), Tris(2-carboxyethyl)phosphine Hydrochloride (TCEP) (1 μ l, 37.5 mM in H₂O), Biotin-N₃ (1 μ l, 15 mM in DMSO) for 1 h at room temperature. The solution was diluted with 100 mM HEPES (pH 7.5) containing 5 mM MgCl₂, and used for HER2 kinase domain labeling without any purification. Recombinant human HER2 (SignalChem, E27-11G) was diluted with 100 mM HEPES buffer containing 5 mM MgCl₂ and used for HER2 kinase domain labeling. Recombinant human HER2 (100 nM) was incubated with labeling reagents (1 μ M) and in the presence or absence of 20 μ M lapatinib for 10 h at 25 °C. Each sample was mixed with 5x SDS-PAGE loading buffer (325 mM Tris-HCl, 15% SDS, 20% sucrose, 0.5 M DTT, and 0.02% BPB, pH 6.8) and incubated for 1 h at room temperature. The samples were applied to SDS-PAGE and electrotransferred onto immune-blot PVDF membranes (Bio-rad), followed by blocking with 5% non-fat dry milk in TBS containing 0.05% Tween (Sigma-Aldrich, St. Louis, MO). The membranes were stained with SAV-HRP (GE Healthcare) or with rabbit HER2/ErbB2 antibody (cell signaling technology, 2242) followed by a HRP-conjugated goat anti-rabbit IgG (Santa Cruz, SC-2004). The membranes were developed with ECL prime western blotting detection system (GE Healthcare).

Chemical labeling of HER2 endogenously expressed in live NCI-N87 cells:

NCI-N87 cells (3.2×10^5 cells) were incubated on 35-mm dishes (BD Falcon) at 37 °C. After 72 hours, the medium was replaced by serum-free DMEM containing labeling reagents with or without lapatinib at 37 °C. After 8 hours, the media was removed, and the cells were washed twice with PBS. Then, the cells were lysed with RIPA buffer (25 mM Tris/HCl, 150 mM NaCl,

1% NP-40, 0.25% DOC, 0.1% SDS, pH7.4) containing protease inhibitor (Calbiochem, 39134), and rotated at 4 °C for 30 min. After centrifugation (10,000 g, 10 min), the supernatant was incubated with Neutra-Avidin agarose resins 50 µL (Thermo scientific, 29202, 50 % slurry) at 4 °C for 1 h to remove endogenously expressing biotinylated protein. To the supernatant were added Cu(MeCN)₄BF₄ (500 µM), BPAA (50 µM), TCEP (250 µM), Biotin-N₃ (100 µM) for 1h at room temperature. Each sample was mixed with 5x SDS-PAGE loading buffer (325 mM Tris-HCl, 15% SDS, 20% sucrose, 0.5 M DTT, and 0.02% BPB, pH 6.8) and incubated for 1 h at room temperature. The samples were applied to SDS-PAGE and western blotting as described above.

Confirmation of chemical labeling of HER2 by immunoprecipitation analysis:

NCI-N87 cells (3.2×10^5 cells) were incubated on 35-mm dishes (BD Falcon) at 37 °C. After 72 hours, the medium was replaced by serum-free DMEM containing labeling reagents with or without lapatinib at 37 °C. After 8 hours, the media was removed, and the cells were washed twice with PBS. Then, the cells were lysed with RIPA buffer (25 mM Tris/HCl, 150 mM NaCl, 1% NP-40, 0.25% DOC, 0.1% SDS, pH7.4) containing protease inhibitor (Calbiochem, 39134), and rotated at 4 °C for 30 min. After centrifugation (10,000 g, 10 min), the supernatant was incubated with Neutra-Avidin agarose resins 50 µL (Thermo scientific, 29202, 50 % slurry) at 4 °C for 1 h to remove endogenously expressing biotinylated protein. To the supernatant were added Cu(MeCN)₄BF₄ (500 µM), BPAA (50 µM), TCEP (250 µM), Biotin-N₃ (100 µM) for 1h at room temperature. The sample was incubated with Herceptin at 4 °C for 12 hour, followed by addition of nProtein A sepharose 4 Fast flow and further incubation at 4 °C for 1 hours. The sepharose was washed 5 times with RIPA. Protein was eluted by addition of 2x SDS-PAGE sample buffer containing 250 mM DTT and boiling in heatblock for 5 min. The samples were applied to SDS-PAGE and western blotting as described above.

Labeling in Live NCI-N87 cells and partial purification of off-target proteins by Fast Protein Liquid Chromatography (FPLC):

Prior to labeling experiments, NCI-N87 cells (2.0×10^6 cells) were incubated on three 10-cm dishes (BD Falcon) at 37 °C. After 72 hours, the medium was replaced by serum-free DMEM containing **2m** (5 µM) at 37 °C. After 8 hours, the media was removed, then lysis buffer cells were washed twice with PBS. Then, the cells were lysed with 50 mM Tris/HCl (pH7.5), 150 mM NaCl, 0.1% Triton containing protease inhibitor (Calbiochem, 39134), and rotated at 4 °C for 30 min. The lysate was centrifuged (10,000 G, 10 min). To the supernatant were added $\text{Cu}(\text{MeCN})_4\text{BF}_4$ (500 µM), BPAA (50 µM), TCEP (250 µM), Biotin- N_3 (100 µM) for 1h at room temperature. The solution was dialyzed against 50 mM Tris/HCl (pH8.0) with a Spectra/Por dialysis membrane (MWCO: 8,000). The resulting solution was purified by FPLC. The chromatographic material was used AKTA purifier (GE Healthcare). Chromatographic separation with the Mono Q 4.6/100 PE column was achieved with a step gradient of B in A at a flow rate of 1.5 ml/min (A. 50 mM HEPES buffer (pH 8.0), B. Buffer A containing 1M NaCl). Mono Q fraction was detected by western blotting as described above. Fraction containing labeled off-target protein was concentrated using a Amicon Ultra-4 (10K, Millipore).

2D-PAGE analysis of off-target proteins:

Partially purified fraction was precipitated using Readyprep 2-D cleanup kit (Bio-rad, 163-2130). The resulting pellet was solubilized Readyprep protein extraction kit (Bio-rad, 163-2086). To the solution was added 0.2 % (w/v) Bio-Lyte 3/10 ampholyte and applied to a 7-cm immobilized pH gradient (IPG) strip, pH 3-10. Rehydration was carried out for 12 hours using a PEOTEAN i12 IEF system, followed by isoelectric focusing (IEF). All IEF equipments and reagents were purchased from Bio-rad. Immediately after IEF, IPG strips were equilibrated with in buffer solution I and II containing 375 mM Tris-HCl (pH 8.8), 6 M urea, 20% glycerol, 2% SDS, and 130 mM DTT for buffer solution I or 137 mM iodoacetamide for buffer solution II. The

equilibrated IPG strips were placed on the top of acrylamide SDS-PAGE minigels, sealed with low melt agarose. After second-dimension separation, proteins were detected by silver staining (Wako) and western blotting.

Identification of off-target proteins by peptide fingerprint analyses:

To identify the labeled proteins, The band pattern of silver stained 2D gel was carefully compared with the corresponding western blot. The labeled protein spots thus identified were excised from the gel, destained, and subjected to in-gel tryptic digestion using in-gel digestion kit (Thermo Scientific). Samples were analyzed by nano-flow reverse phase liquid chromatography followed by tandem MS, using a Triple TOF 5600+ (AB SCIEX, Concord, Canada).

A capillary reverse phase HPLC-MS/MS system composed of an Eksigent Ekspert nano-LC 400 HPLC system (AB SCIEX) which was directly connected to an AB SCIEX quadrupole time-of-flight (QqTOF) TripleTOF 5600+ mass spectrometer (AB SCIEX) in Trap and Elute mode. In the Trap and Elute mode, samples were automatically injected using Ekspert 400 system into a peptide trap column (ChromeXP, C18-CL, 200 μ m I.D. x 0.5 mm, 3 μ m particle size, 120 Å pore size, AB SCIEX) attached to a cHiPLC system (AB SCIEX) for desalinating and concentrating peptides. After washing the trap with MS-grade water containing 0.1% trifluoroacetic acid and 2% acetonitrile (solvent C), the peptides were loaded into a separation capillary reverse phase column (ChromeXP, C18-CL, 75 μ m I.D. x 150 mm, 3 μ m particle size, 120 Å pore size, AB SCIEX) by switching the valve. The eluents used were: A, 100% water containing 0.1% formic acid, and B, 100% acetonitrile containing 0.1% formic acid. The column was developed at a flow rate of 0.3 μ L/min with the concentration gradient of acetonitrile: from 2% B to 32% B in 20 min, 32% B to 80% B in 1 min, sustaining 80% B for 10 min, from 80% B to 2% B in 1 min, and finally re-equilibrating with 2% B for 15 min. Mass spectra and tandem mass spectra were recorded in positive-ion and “high-sensitivity” mode with a resolution of ~35,000 full-width half-maximum. The nanospray needle voltage was typically 2,300 V in

HPLC-MS mode. After acquisition of ~ 5 samples, TOF MS spectra and TOF MS/MS spectra were automatically calibrated during dynamic LC-MS & MS/MS autocalibration acquisitions injecting 50 fmol BSA. Analyst TF1.6 system (AB SCIEX) was used to record peptide spectra over the mass range of m/z 400–1250, and MS/MS spectra in information-dependent data acquisition over the mass range of m/z 100–1600. For CID-MS/MS, the mass window for precursor ion selection of the quadrupole mass analyzer was set to 0.7 ± 0.1 Da. The precursor ions were fragmented in a collision cell using nitrogen as the collision gas. Advanced information dependent acquisition (IDA) was used for MS/MS collection on the TripleTOF 5600+ to obtain MS/MS spectra for the 10 most abundant parent ions following each survey MS1 scan (250 msec acquisition time per MS1 scan, and typically 100 msec acquisition time per each MS/MS). Dynamic exclusion features were based on value m/z and were set to an exclusion mass width 50 mDa and an exclusion duration of 12 sec.

Searches were performed by using the Mascot server version 2.4.0 (Matrix Science, MA, USA) against latest Swissprot database for protein identification. Searching parameters were set as follows: enzyme selected as used with three maximum missing cleavage sites, species limited to human, a mass tolerance of 40 ppm for peptide tolerance, 0.1 Da for MS/MS tolerance, variable modification of carbamidomethyl (C), oxidation (M) and chemical modification (C, E, H, K, S, T, Y). The maximum expectation value for accepting individual peptide ion scores [$-10 \cdot \log(p)$] was set to ≤ 0.05 , where p is the probability that the observed match is a random event. Protein identification and modification information returned from Mascot were manually inspected and filtered to obtain confirmed protein identification and modification lists of CID MS/MS.

Immunoprecipitation analysis of off-target protein:

NCI-N87 cells (3.2×10^5 cells) were incubated on 35-mm dishes (BD Falcon) at 37 °C. After 72 hours, the medium was replaced by serum-free DMEM containing labeling reagents with or without lapatinib at 37 °C. After 8 hours, the media was removed, and the cells were washed

twice with PBS. Then, the cells were lysed with RIPA buffer (25 mM Tris/HCl, 150 mM NaCl, 1% NP-40, 0.25% DOC, 0.1% SDS, pH7.4) containing protease inhibitor (Calbiochem, 39134), and rotated at 4 °C for 30 min. After centrifugation (10,000 g, 10 min), the supernatant was incubated with Neutra-Avidin agarose resins 50 μ L (Thermo scientific, 29202, 50 % slurry) at 4 °C for 1 h to remove endogenously expressing biotinylated protein. To the supernatant were added $\text{Cu}(\text{MeCN})_4\text{BF}_4$ (500 μ M), BPAA (50 μ M), TCEP (250 μ M), Biotin- N_3 (100 μ M) for 1h at room temperature. The sample was incubated with rabbit PDI antibody (cell signaling technology, 2446) at 4 °C for 18 hour, followed by addition of nProtein A sepharose 4 Fast flow and further incubation at 4 °C for 1 hours. The sepharose was washed 5 times with RIPA. Protein was eluted by addition of 2x SDS-PAGE sample buffer containing 250 mM DTT and boiling in heatblock for 5 min. The samples were applied to SDS-PAGE and western blotting as described above.

Reciprocal immunoblot of 2D-PAGE western blots:

To further confirm the labeled protein by **2m**, the 2D-PAGE westernblots with SA ν -HRP were reprobed by a standard procedure. In brief, the membrane was washed with stripping buffer (25 mM glycine-HCl, pH 2.0 containing 1.0% SDS) with shaking. After 30 min, the stripping buffer was replaced with PBS and the membrane was washed twice with PBS for 10 min. The membranes were stained with rabbit PDI antibody (cell signaling technology, 2446) followed by a HRP-conjugated goat anti-rabbit IgG (santa cruz, SC-2004).

Chemical labeling of recombinant PDI in test tube:

Rhodamine tethered labeling reagent **2m-Rh** was prepared from alkyne labeling reagents **2m** by click chemistry in test tube. Click chemistry condition were as follows: to a stirred solution of alkyne labeling reagents (1 μ L, 5 mM) was added $\text{Cu}(\text{MeCN})_4\text{BF}_4$ (1 μ L, 75 mM in DMSO), bathophenanthroline disulfonic acid (BPAA) (1 μ L, 7.5 mM in DMSO : H_2O = 1:1),

Tris(2-carboxyethyl)phosphine Hydrochloride (TCEP) (1 μ l, 37.5 mM in H₂O), Rhodamine-N₃ (5 μ l, 3.3 mM in DMSO) for 1h at room temperature. The solution was diluted with 50 mM HEPES (pH 7.4) containing 3 mM DTT, and used for PDI labeling without any purification. Recombinant human protein disulfide isomerase (44 μ M, R&D systems, 4236-DI) was diluted with 50 mM HEPES (pH 7.4) containing 3 mM DTT, and used for PDI labeling. Recombinant human protein disulfide isomerase (220 nM) was incubated with labeling reagents in the presence or absence of 20 μ M lapatinib or 20 μ M estradiol for 18 h at 25 °C. Each sample was mixed with 5x SDS-PAGE loading buffer (325 mM Tris-HCl, 15% SDS, 20% sucrose, 0.5 M DTT, and 0.02% BPB, pH 6.8) and incubated for 1 h at room temperature. The samples were subjected to SDS-PAGE and detected by in-gel fluorescence image. After fluorescence imaging, the gels were stained by silver stain.

Insulin turbidity assay for PDI activity:

PDI activity was assessed by measuring the PDI-catalyzed reduction of insulin in the presence of DTT. Reduction-induced aggregation of insulin chain was monitored in a 96-well plate at 650 nm, using microplate reader (Tecan, infinite 200). The incubation mixture contained 100 mM NaH₂PO₄ (pH 7.0), 1 mM DTT, 2 mM EDTA, 1.0 mg/ml bovine insulin (Sigma), and 830 ng/ml Recombinant human PDI (R&D systems, 4236-DI).

3-5. References.

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List of Publications

Chapter 1. Construction of protein-based biosensor using ligand-directed chemistry for detection of ligand binding event.

Kei Yamaura, Shigeki Kiyonaka, Itaru Hamachi.

Manuscript in preparation. **(Chapter 1)**

Chapter 2. Discovery of new allosteric modulators for GABA_A receptor by ligand-directed chemistry.

Kei Yamaura, Shigeki Kiyonaka, Tomohiro Numata, Ryuji Inoue,
Itaru Hamachi.

Nat. Chem. Biol. Accepted. **(Chapter 2)**

Chapter 3. Live cell off-target identification of lapatinib using ligand-directed tosyl chemistry.

Kei Yamaura, Keiko Kuwata, Tomonori Tamura, Yoshiyuki Kioi,
Yousuke Takaoka, Shigeki Kiyonaka, Itaru Hamachi.

Chem. Commun. **50**, 14097-14100 (2014). **(Chapter 3)**

List of Presentations

Domestic Symposium

1. In cell chemical labeling (1) Specific labeling of HER2, a breast cancer marker using LDT chemistry

Kei Yamaura, Tomonori Tamura, Yoshiyuki Kioi, Yousuke Takaoka, Shigeki Kiyonaka, Itaru Hamachi

93th Annual Meeting of Chemical Society of Japan, Aichi, March 2013.

2. *Off-target Identification of Lapatinib, anti-HER2 drug Using Ligand-Directed Tosyl Chemistry*

Kei Yamaura, Tomonori Tamura, Keiko Kuwata, Yoshiyuki Kioi, Yousuke Takaoka, Shigeki Kiyonaka, Itaru Hamachi

7th Symposium on Biorelevant Chemistry, Aichi, September 2013.

3. *Chemical biology of nerve synapse (3) Chemical labeling of inhibitory neurotransmitter receptors, GABA_A receptors*

Kei Yamaura, Shigeki Kiyonaka, Itaru Hamachi

95th Annual Meeting of Chemical Society of Japan, Chiba, March 2015.

4. *Protein Labelling Using Ligand-Directed Chemistry (6) Chemical labeling of GABA_A receptors for a drug screening assay*

Kei Yamaura, Shigeki Kiyonaka, Itaru Hamachi

96th Annual Meeting of Chemical Society of Japan, Kyoto, March 2016.