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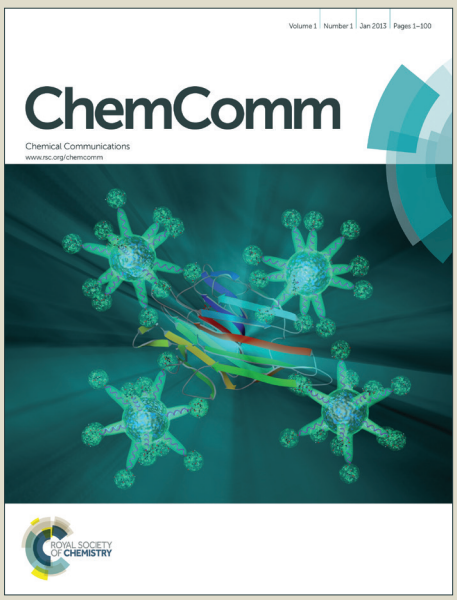

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Live Cell Off-target Identification of Lapatinib Using Ligand-Directed Tosyl Chemistry

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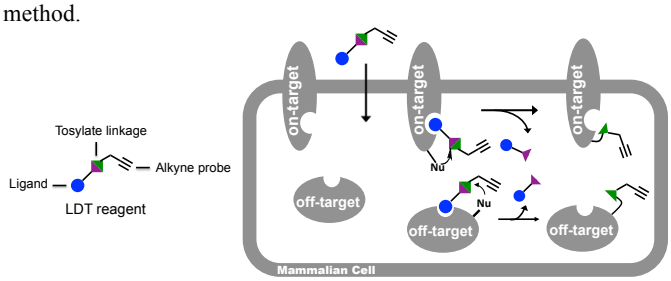
We describe ligand-directed tosyl (LDT) chemistry is applicable to off-target identification in live cells. Lapatinib (Lap)-based LDT reagents labeled not only a receptor tyrosine kinase, HER2, target protein, but also protein disulfide isomerase (PDI) that should be an off-target protein for Lap.

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] Therefore, identification of such off-target 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.[2, 3] 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 sometimes excludes weak but specific interaction with off-targets. Recently, although some of the covalent trapping based methods were proposed,[4, 5] these are still insufficiently developed to date.

We recently invented ligand-directed tosyl (LDT) chemistry for covalent labeling of endogenous proteins, which can be applied under live cell conditions.[6] 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 probe 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). Compared with photo-crosslinking, covalent labeling occurs without any assistance such as UV-light irradiation. In contrast to activity-based methods, non-covalently interacting ligand-protein partners can be trapped by the LDT method.



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

For the proof-of-principle experiments, we employed lapatinib (Lap),[7] 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,[8] we 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 our group and new compounds were well characterized by NMR and high-resolution mass spectrometry.

Prior to live cell study, we initially confirmed the labeling efficiency of these reagents for on-target protein (HER2) in test tube. The biotin tethered labeling reagents (**1*m*-Bt, 1*p*-Bt, 2*m*-Bt, 2*p*-Bt, 3*m*-Bt, 3*p*-Bt**), which were converted from the corresponding alkyne-type of LDT reagents (**1*m*, 1*p*, 2*m*, 2*p*, 3*m*, 3*p***) by click chemistry (ESI,† Scheme S1),[9] 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 Fig. S1, ESI†, the labeling efficiency is greatly dependent on the spacer length, that is, the shortest one (**1*p*-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.

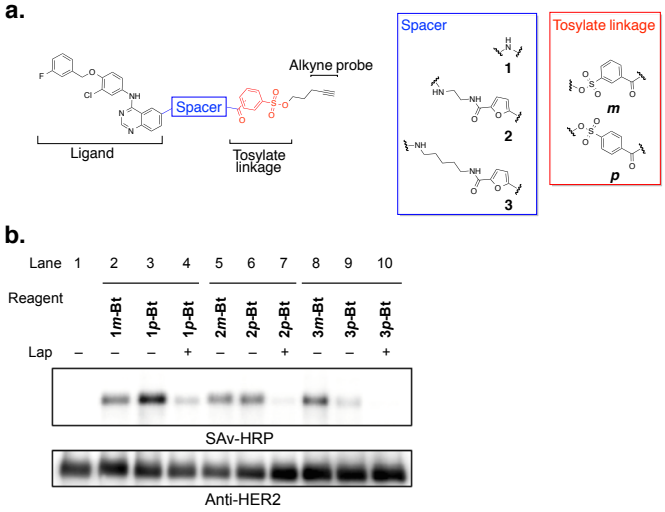


Fig. 1 Molecular structures of LDT reagents and HER2 labeling *in vitro*. (a) LDT reagents in this study. (b) *In vitro* HER2 kinase domain labeling. The reaction was carried out with 100 nM HER2 kinase domain and 1 μM biotin tethered labeling reagents in the presence or absence of 20 μM Lap in HEPES buffer (100 mM HEPES, 5 mM $MgCl_2$, pH 7.5) at 25 °C for 10 h. Upper, biotin-blotting analysis using streptavidin (SAv)-HRP. Lower, western blotting analysis using rabbit anti-HER2 antibody.

Having these labeling reagents in hand, we 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 (**1*m*, 1*p*, 2*m*, 2*p***) in the cell culture medium, lysis followed by click reaction to tether the biotin unit, the labeling reaction was evaluated by WB analysis (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 **1*p*** 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 which showed the stronger band by biotin blotting (Fig. S2, ESI†). The similar HER2 labeling occurred in a HER2-positive human breast cancer cell line, SKBR3 (Fig. S3, ESI†) but not in other HER2-negative cancer cell lines such as MCF7 (data not shown), implying that the LDT reagent **1*p*** is a powerful tool for selective labeling of intracellular kinase domain of HER2 in live cell conditions.

Interestingly, we noticed another band around 55 kDa appeared in the WB analyses in the gastric and breast cancer cell lines (Fig. 2a and Fig. S3, ESI†). In gastric NCI-N87 cells, at the lower concentration of the labeling reagent (**1*p***), the labeled HER2 band was predominantly detected, whereas the 55 kDa band slightly appeared in its intensity by the increment of **1*p*** (Fig. 2b). 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. 2a, this off-target band intensity was again varied with the labeling reagents, that is, **2*m*** produced the more intense band than **1*m***, **1*p*** and **2*p***. 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 **2*m*** concentration and the labeling time (Fig. S4, S5, ESI†), 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.

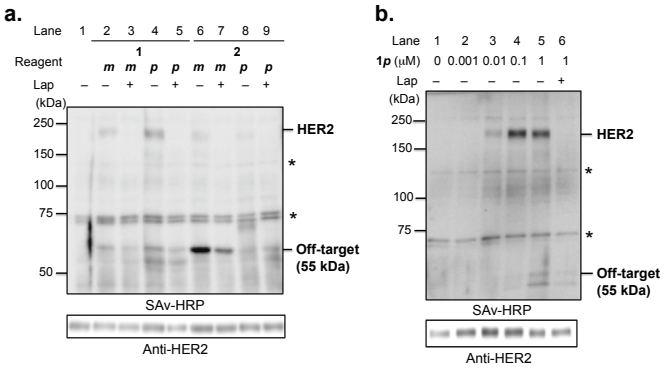


Fig. 2 Endogenous HER2 labeling in live NCI-N87 cells. (a) Chemical labeling of HER2 in live NCI-N87 cells by LDT reagents. NCI-N87 cells were treated with 5 μM labeling reagents in serum free DMEM at 37 °C for 8 h with or without 20 μM Lap. * indicates biotinylated proteins endogenously expressed in NCI-N87 cells (b) Concentration-dependent labeling of HER2 by **1*p***. Chemical labelings were conducted with **1*p*** under the same conditions as in (a). The blotting indicates that 0.1 μM of **1*p*** is enough for the HER2 binding. High concentration of **1*p*** (> 1 μM) decreased HER2 labeling (data not shown). Upper, biotin-blotting analysis. Lower, western blotting analysis using anti-HER2 antibody.

Subsequently, we sought to identify the off-target protein. 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. 3a). The gel spot was excised, treated with in-gel trypsin digestion and then the resultant peptide fragments were determined by MSMS analysis (TripleTOF 5600+, AB SCIEX). The MASCOT analysis of the obtained MS data (the protein score 7311 and the coverage 75%, Table S1, ESI†) revealed that the off-target protein was protein disulfide isomerase (PDI), an intracellular enzyme that catalyzes disulfide bond rearrangement from the mis-linked bond to the correct one for native protein folding,[10] and PDI has been regarded as a potential therapeutic target for cancer and neurodegenerative diseases.[11] 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. 3b). The IP experiment using PDI antibody finally confirmed the off-target protein was PDI (Fig. S6, ESI†). In addition, the PDI labeling by a rhodamine tethered labeling reagent **2*m*-Rh** in test tube was examined using purified PDI (Scheme S2, ESI†). The fluorescent gel image analysis showed that PDI was indeed labeled by **2*m*-Rh** in a dose-dependent manner (Fig. 4a). More importantly, the labeling was clearly inhibited by addition of 20 μM of Lap (Fig. 4b (lanes 2 and 3), Fig. S7, ESI†) and the inhibition occurred in the concentration dependent manner (Fig. S8, ESI†).

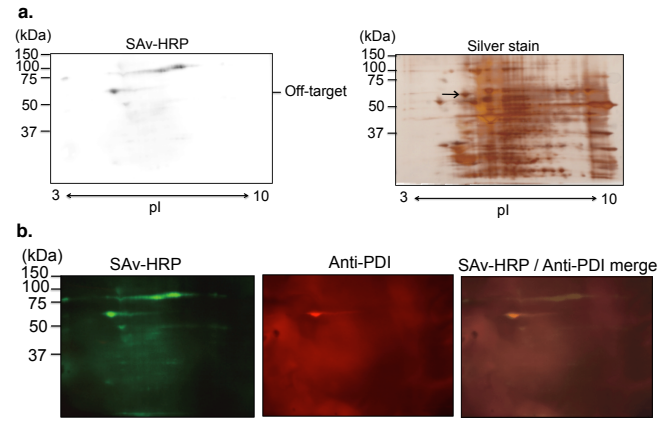


Fig. 3 Off-target identification of **2*m*** in live NCI-N87 cells. (a) 2D-PAGE silver staining and biotin-blotting of roughly purified cells lysate from NCI-N87 labeled with **2*m***. Left, biotin-blotting analysis. Right, silver staining. (b) Reciprocal 2D-PAGE immunoblot analysis of labeled proteins by **2*m*** using anti-PDI antibody. Pseudo-color images are shown. 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.

It was reported that PDI promiscuously binds steroid hormones such as estradiol (E2) mainly through hydrophobic interactions to regulate PDI activity.[12-14] In practice, we found that PDI labeling by **2*m*-Rh** was suppressed by addition of E2 as well as Lap *in vitro*, suggesting that Lap binding to PDI may compete with E2 (Fig. 4b, lanes 2−4, Fig. S7, ESI†). In order to explore the biological relevance of Lap-PDI interaction, we next examined the impact of Lap (or E2) on the PDI activity by the conventional insulin turbidity assay (Fig. 4c, Fig. S9, ESI†).[13] As previously reported,[12] 1 μM of E2 decreased the initial rate for the insulin aggregation catalysed by PDI (Fig. 4c, Fig. S10, ESI†). 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. S11, ESI†). This Lap-induced activity recovery of PDI showed the clear concentration dependence from 1 μM to 10 μM, 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.[12b] which let us expect another possibility that the competitive binding of Lap releases E2 from PDI, resulting in the activation of hormonal receptors in cells.

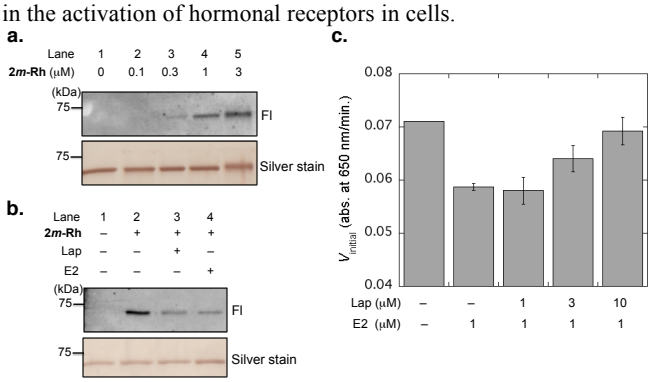


Fig. 4 Effect of Lap on PDI labelling by **2*m*-Rh** and PDI activity. (a) SDS-PAGE analysis of PDI labeling by in gel fluorescence imaging (Fl) and silver staining. (a, b) Fluorescent (upper) and silver stain (lower) gel images of recombinant PDI labeled by **2*m*-Rh**. In (a), the reaction was carried out with 220 nM PDI and each concentration of **2*m*-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 μM Lap and 20 μM E2 at 25 °C for 18 h. [**2*m*-Rh**] = 2.2 μM. (c) 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 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 in the presence or absence of 1 μM E2 and / or each concentration of Lap at 25 °C.

In summary, we 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, we found PDI as a potential off-target of Lap, a FDA-approved anticancer drug for the first time. The binding affinity of Lap to PDI was estimated to be microM range, which highlighted an advantage of moderate reactivity in LDT labeling method, that is, a relatively weak interaction partner can be picked up by covalent labeling under live cell conditions. On the basis of the subsequent activity assay, we 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.[15] However, screened proteins in this analysis are generally limited in kinase family only. In contrast, all cellular proteins are examined in our approach under live cell conditions. Moreover, ligand moiety can be removed after labeling in the LDT method,[6] which may allow functional analyses of labelled off-target proteins. We 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.

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† Electronic Supplementary Information (ESI) available: Experimental details, figures (Fig. S1–S11), schemes (Schemes S1 and S2), and a table (Table S1). See DOI: 10.1039/c000000x/

‡ Dedicated to Professor Seiji Shinkai on the occasion of his 70th birthday.

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