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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 is 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. 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. 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, 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. 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.

For the proof-of-principle experiments, we employed lapatinib (Lap), 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, 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 (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 (ESI, Scheme S1)\textsuperscript{3} 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, ES†, the labeling efficiency is greatly dependent on the spacer length, that is, the shortest one (1p-Rh) 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.

Interestingly, we noticed another band around 55 kDa appeared in its intensity by the increment of spacer length and the orientation of tosyl unit. More importantly, the labeling was clearly inhibited by addition of 20 µM of Lap (Fig. 1b) and 20 µM of Lap (Fig. S4, S5, ES†), 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.

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-tethered labeling reagents in the presence or absence of 20 µM Lap in HEPES buffer (100 mM HEPES, 5 mM MgCl₂, 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.

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**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 MgCl₂, 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.

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 MS analysis (TripleTOF 5600+, AB SCIEX). The Mascot analysis of the obtained MS data (the protein score 7311 and the coverage 75%, Table S1, ES†) 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, and PDI has been regarded as a potential therapeutic target for cancer and neurodegenerative diseases. 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, ES†). In addition, the PDI labeling by a rhodamine tethered labeling reagent 2m-Rh in test tube was examined using purified PDI (Scheme S2, ES†). The fluorescent gel image analysis showed that PDI was indeed labeled by 2m-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, ES†) and the inhibition occurred in the concentration dependent manner (Fig. S8, ES†).
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 2m-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, ESIF). 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, ESIF).\[15\] As previously reported,\[12\] 1 mM of E2 decreased the initial rate for the insulin aggregation catalysed by PDI (Fig. 4c, Fig. S10, ESIF). 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, ESIF). 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 might 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,\[15\] 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.

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-targerts is facilitated.

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

Notes and references


