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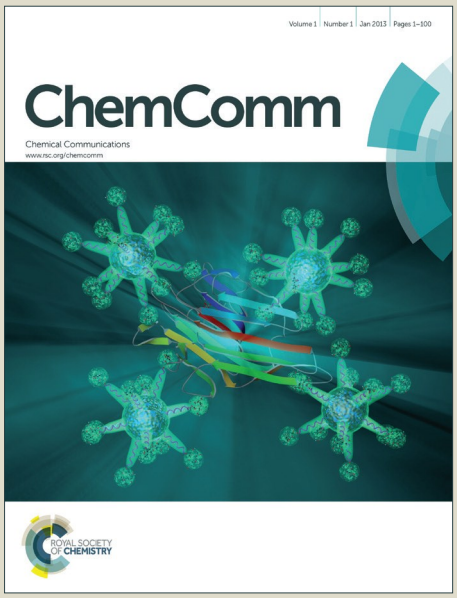

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View Article Online
DOI: 10.1039/C6CC04030F

Journal Name

COMMUNICATION

Focusing on probe-modified peptides: a quick and effective way for target identification

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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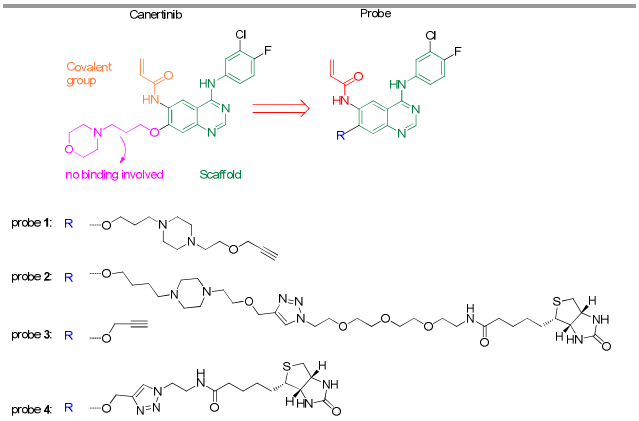
A new efficient method focusing on probe-modified peptides was developed to identify the target protein and modification site of a hit compound or a drug. This method exhibited high click conjugation efficiency and few false-positive results. The modification site further facilitated target validation, biological mechanism study and new indications exploration.

Covalent inhibitor is widely used in the drug discovery[1] for desirable features, including high efficiency of target disruption[2] and long duration of action[3] because of the covalent modifications of target proteins by the drug. Meanwhile, the chemically reactive group of covalent drugs may also interact with other targets besides the desired one, which could lead to beneficial usages for new indications or cause side effects.[4] However, there is no easy way to know which target interacts with the drug causing those side effects, instead most side effects or new indications are passively discovered during clinical observation. Activity-based protein profiling (ABPP) is a technology that, in principle, can define all targets irreversibly modified by covalent inhibitors.[5] Introducing click chemistry (CC) into ABPP further expanded its application scope as adding small-sized clickable group on probes might not affect the binding affinity much,[6] conversely immobilizing the active probe onto a resin often diminishes the binding affinity.[7]

CC-ABPP has been used for characterizing potential targets of covalent drugs such as Orlistat[8] and Dasatinib[9]. However, high background disruption in CC-ABPP, giving many false positives, has been troublesome due to the highly cumbersome and time-consuming process of discrimination of the real target from many specious candidates. A key feature of using a covalent probe is that the probe could irreversibly modify its targeting proteins. Ideally, discovery of the probe-modified peptide(a piece of the protein) would reveal both the identity of target protein and the location of binding pocket by decrypting the sequence of the peptide and might further reveal the possible mechanism of the action of the probe.[10] More importantly, these information would greatly facilitate the target validation process.[11] For this purpose, we aimed to develop a BTC-ABPP method based on ABPP, by converting the reactants of click conjugation reaction from proteins to peptides (tantamount to Biochemistry To Chemistry, BTC), to identify the modified peptides. Furthermore, this method might also effectively improve the efficiency of click conjugation and the affinity enrichment process. The new method could be a useful tool paralleled with traditional ABPP.

Protein kinases have been widely selected as therapeutic targets for developing anti cancer drugs, and a few covalent inhibitors have been developed and approved by FDA. Here, we selected HER2 kinase and the known irreversible inhibitor Canertinib as our model system, to develop the BTC-ABPP method. Based on the analysis of Canertinib, the acrylamide moiety could form a covalent bond with the Cysteine 805 residue of HER2; and structural modifications at 7-propoxymorpholine did not significantly alter its inhibition potency to HER2 kinase activity.[11] Accordingly, we designed



Scheme 1 Design and synthesis of probe **1-4**

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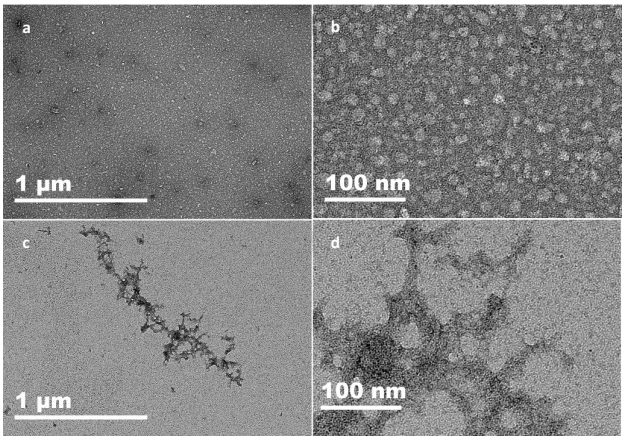


Figure 1. Negative stain electron micrographs. (a, b), Images of native cell lysates in RIPA lysis buffer with 2% uranyl acetate staining. (c, d), Images of denatured cell lysates dissolved in 1% SDS PBS buffer with 2% uranyl acetate staining. Scale bars: 1 μm (left) and 100 nm (right).

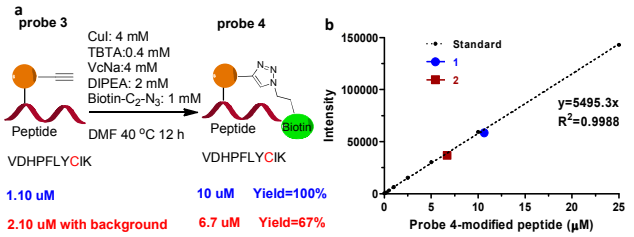
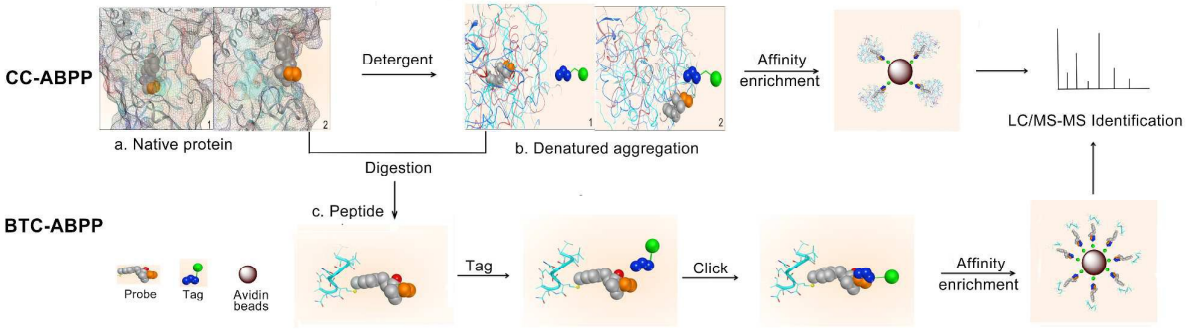


Figure 2 The click chemistry conjugation yields for reactions at the peptide level. a) Probe **3**-modified peptides were clicked to a biotin tag (Biotin-C_2-N_3) in DMF solvent buffer. For the complex system, 3 mg/ml of cell lysate peptides were added to the reaction buffer as an artificial background. b) A standard concentration curve of product probe 4-modified peptides was generated from data acquired via TSQ Quantum LC-MS/MS analysis

and synthesized four probes (see Scheme 1) with either clickable groups or biotin tags coupled on the side chain of Canertinib. IC_{50} values of four probes were comparable in the HER2 kinase assay (Figure S1).

Scheme 2 illustrated differences between CC-ABPP and BTC-ABPP. In CC-ABPP, the click conjugation step is usually carried out in cell lysates for linking a reporter tag to the protein-bound probe. The clickable group on the probe sitting in the binding pocket is often difficult to reach or unreachable for the multiple components (Cu(I)\ligand\azide or alkyne)[12] of the click conjugation reaction (Scheme 2a). Protein denaturation destroys the binding pocket and releases the probe. However, the massively high concentration of proteins in the cell lysates could potentially foster messy interference among the proteins, and form large intertwined protein aggregation.[13] In electron microscopy (Figure 1), the pattern of proteins of the native cell lysates was relatively homogenous, while the pattern of proteins of the denatured cell lysates was highly aggregated. We mesured the size of particles of both samples by quasielastic light scattering and results also showed the denatured cell lystates had bigger sizes of particals than the native cell lysates (Figure S2). The protein aggregation could then create other types of steric hindrances around the probe (Scheme 2b),which might lower efficiency of the click reactions. Furthermore, probe-modified target proteins would also be in an aggregated form, rather than in a free form. This would complicate subsequent pull-down and separation steps, thereby worsing the high background problem. Digestion of proteins into peptides should remove the steric hindrances, therefore facilitate click conjugation (Scheme 2c). Further, peptides are small molecules and can be dissolved easily in DMSO, DMF, methanol, and other organic solvents. These characteristics of peptides not only sustain the kinetic energy needed for click reaction,[14] but also avoid buffer compatibility issues that are problematic at the protein level, especially for membrane proteins.[15]

To prove this idea, we first tested the efficiency of the click reaction between a probe **3**-modified peptide (VDHPFLYCIK) and a biotin tag (Biotin-C_2-N_3) in an organic solvent (DMF). Based on TSQ Quantum LC-MS/MS analysis, a 100% yield of click product—probe **4**-modified peptide was formed within 12 h in the presence of the TBTA catalyst (Figure 2). Thereafter, same reaction was carried out in a mixture of peptides digested from cell lysates. Without any optimization to the reaction conditions, a high 67% yield of the product was detected. The efficiency of click conjugation at the protein level and at the peptide level was also compared(Figure S3a). The yield in peptides mixture digested from recombinant HER2 was 3 times higher than in denatured recombinant HER2



Scheme 2 Schematic illustrations of CC-ABPP and BTC-ABPP for new target identification. a). Illustration of environment nearby the probe after covalently binding to target protein: 1). The click conjugation is sterically prohibited; 2). The click conjugation is sterically possible; b). Illustration of environment nearby the probe after proteins denaturation: 1). The caged probe is unreachable for the click conjugation; 2). The exposed probe is reachable for the click conjugation. c). In new BTC-ABPP, the digested probe-linked peptides are favorable reactants for click conjugation.

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solution (Figure S3c,d). Moreover, adding cell lysates (10 times amount of HER2) to each sample enlarged the difference to nearly 5 times (Figure S3e,f). The results indicated that the change of reactants from proteins to peptides could provide high efficiency of click chemistry, tantamount to transition from a biochemical system to a chemical system (BTC).

BTC-ABPP method is designed to specifically enrich probe-modified peptides through affinity purification steps. It is known that MS/MS signals of a peptide might be affected or interrupted by chemical modifications.[16] The behaviors of the probe-modified peptides in LC-MS/MS analysis should be clarified first for defining the probe modification. For this reason, we synthesized and analyzed a probe **2**-modified peptide and a probe **4**-modified peptide (Figure S4a). A PEG chain on probe **2** was designed to increase its solubility in polar solvents like water and acetonitrile, which are frequently used during LC-MS analysis. The MS/MS results of the probe **2**-modified peptide indicated that many high-density ion signals were derived from broken PEG chains, which greatly suppressed the fragment peaks formed by the peptides (Figure S4b,c). To avoid the instability issue of linker chain, the PEG chain was removed and probe **4** was designed. Probe **4**-modified peptide was very stable (Figure S4d), and MS/MS signals matched well with the modified peptides. Thus it is prudent to study the behavior of probe-modified peptides in MS/MS analysis prior to conducting BTC-ABPP for an unknown target identification.

Next, we used probe **4** and recombinant HER2 protein to evaluate the BTC-ABPP method. We first confirmed that Canertinib could covalently modify the recombinant HER2 (Figure S5), and the western blot of biotin showed a dose-dependent labeling efficiency of biotin-linked probe **4** (Figure S6). Considering protein digestion by proteases might produce different probe-modified peptides, three proteases

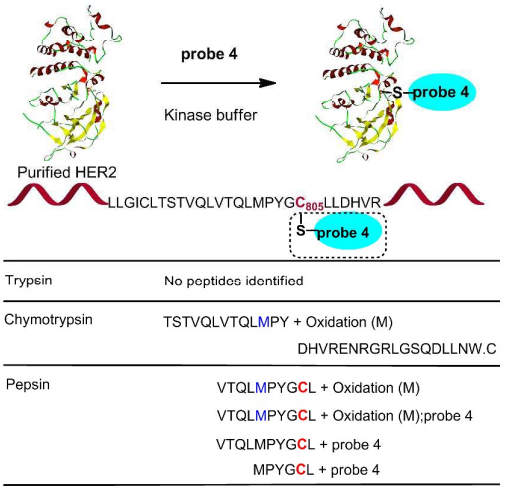

Figure 3 Characterization of the probe-modified peptides of recombinant HER2. MS/MS identification of peptides resulted from the digestion of HER2 by different proteases. Cysteine 805 is marked in bold red.

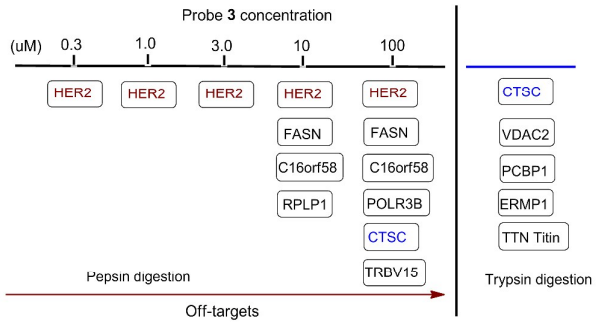

Figure 4 Concentration-dependent proteomics analysis for probe **3** in SK-Br3 cells. Cells were treated with different concentrations of probe **3** (0.3-100 μM, 4 h), followed by digestion with pepsin and trypsin(only 100 μM),click chemistry with Biotin-C_2-N_3 tags, enrichment, and analysis with LC-MS/MS.

including trypsin, chymotrypsin, and pepsin were used to digest the probe **4**-modified HER2 respectively. No probe-modified peptides were found in the samples digested by trypsin or chymotrypsin, but two fragments of probe-modified peptides were found in the pepsin-digested sample (Figure. 3).The results could be explained as different proteases hydrolysed a protein at its specific sites, which might lead to a modified peptide either too long and too hydrophobic to dissolve during LC-MS analysis, or too short (a peptide less then 3 amino acids) to give meaningful information about the target protein. These experiments suggested that it is necessary to use more than one type of the common proteases[17] to digest the cell lysates when dealing with unknown targets, so that the chance of missing a true target is minimized.

Following successfully identified the probe-modified peptides in HER2 kinase *in vitro*, we performed BTC-ABPP in living cells to explore whether the target, HER2, and other potential targets could be efficiently identified. We treated SK-Br3 cells with probe **3** at different concentrations (from about 1×IC_{50} to 300×IC_{50}). After 4 h, the cells were collected, lysed, and digested by pepsin. The probe **3**-modified peptides were conjugated to a biotin tag (Biotin-C_2-N_3) via click chemistry. After the excess biotin tags were removed by dialysis (Figure S7), the probe-modified peptides were enriched with streptavidin beads, washed extensively, and then eluted and analyzed with LC-MS/MS. As shown in Figure 4, the probe-modified peptide MPYG**C_{805}**L from HER2 was successfully detected at all of the five concentrations. HER2 was the only target detected when the probe was used at a concentration lower than 10×IC_{50}. When higher concentration of the probe was used, other potential target proteins including Cathepsin C (CTSC, also known as dipeptidyl peptidase I) were identified. CC-ABPP was also performed in living cells using probe **3** (Figure S8) and the candidates list based on Mascot score (Table S1) was compared with the list of BTC-ABPP (Table S2). As mentioned previously, it is necessary to use more than one protease for the digestion in parallel to avoid missing target information. So the cell lysates were also digested by trypsin, followed by click conjugation and enrichment, and a

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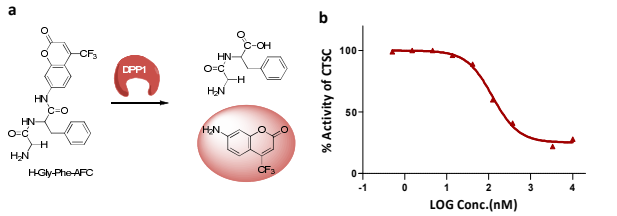

Figure 5 a) The mechanism of CTSC enzymatic assay; b) Canertinib exhibited dose-dependent inhibition to human CTSC enzyme activity.

new list of candidates was generated after MS analysis. In these two experiments, a total of 11 candidates were detected through the probe-modified peptides, which also revealed the modification site on the target proteins (Figure S9).

Benefiting from BTC-ABPP, finding the modification site of the probe can reveal its possible mechanisms of action. For example, CTSC, one candidate identified both in the trypsin digestion and in the pepsin digestion, was modified by Canertinib probe at Cys_{234}. Many CTSC inhibitors are irreversible inhibitors that covalently bind to Cys_{234} residue in the catalytic site[19], indicating Canertinib as a potential CTSC inhibitor. Thus we developed CTSC enzymatic assay according to the previous report(Figure 5a).[20] Canertinib exhibited dose-dependent inhibition to CTSC activity, with an IC_{50} of 0.12 μM (Figure 5b). CTSC is involved in the activation of granule-associated serine proteases and is also associated with many inflammatoryand autoimmune diseases such as Rheumatoid Arthritis and Chronic Obstructive Pulmonary Disease (COPD).[18] Most known CTSC inhibitors are analogs of the dipeptide substrates, but few can enter clinical trials due to poor metabolic stability. Now Canertinib can be used as a new parent nucleus to develop novel CTSC inhibitors which will facilitate the drug discovery process.

In conclusion, we have developed a new method based on ABPP technology by converting the reactants from proteins to peptides, and changing the manipulation system from biochemistry to chemistry. This method can both improve the efficiency of click conjugation reaction and reduce false-positive targets. Besides, under the guidance of the information encoded by the probe-modified peptide (ID of target and the modification site), BTC-ABPP can effectively discriminate the functional target from other unrelated candidates, and accelerate the target validation process. Thus, the BTC-ABPP method should be a good alternative to the known ABPP technology, and will benefit the inevitable discovery of biologically improtant targets and facilitate the mechanism study.

We thank Dr. Wanzhong He for assistance with the electron micrographs. We acknowledge the National High Technology Project 973 (2011CB504300). We thank Prof. Zhenhua Zhang (China Agricultural University) for providing several azide reagents (National Key Technologies R&D Program of China, 2015BAK45B01, CAU).

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