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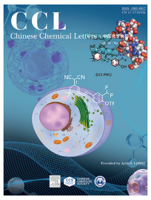

# Isotope-labeled click-free probes to identify protein targets of lysine-targeting covalent reversible molecules

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## ABSTRACT

Recent advances in drug development and bioactive molecules that covalently target lysine residues have shown substantial progress. Both reversible and irreversible covalent inhibitors are developed for targeting lysine residues. The identification of protein targets and binding sites of these lysine-targeting molecules in the whole proteome is crucial to understand their proteome-wide selectivity. For covalent inhibitors, the pull down-based methods including activity-based protein profiling (ABPP) are commonly used to profile their target proteins. For covalent reversible inhibitors, it is not easy to pull down the potential protein targets as the captured proteins may get off beads because of the reversible manner. Here, we report a pair of isotope-labelled click-free probes to competitively identify the protein targets of lysine-targeting covalent reversible small molecules. This pair of isotopic probes consists of a lysine-reactive warhead, a desthiobiotin moiety and isotopicable linker. This integrated probe could eliminate the background proteins induced by the click chemistry during the pull-down process. To demonstrate the feasibility of our newly-developed probes for the protein target identification, we selected the natural product Gossypol in that we proved for the first time that it could modify the lysine residue in a covalent reversible manner. Finally, we confirmed that this pair of integrated probes can be used in a competitive manner to precisely identify the protein target as well as binding sites of Gossypol. Interestingly, pre-treatment of Gossypol could stop the antibody from recognizing Gossypol-binding proteins. Together, our isotope-labeled click-free probes could be used for whole-proteome profiling of lysine-targeting covalent reversible small molecules.

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Lysine is found at crucial functional sites, including enzyme active sites [1] and protein-protein interaction interfaces [2]. For instance, K258 locating in the active site of pyridoxal phosphate (PLP) enzyme [3] facilitates the transaminase activity. K49 on the surface of LC3-interaction regions is a site of post-translational modifications that could disrupt the interaction capability between LC3 protein and its interacting partner [4]. In addition, the positively-charged side chain of lysine can participate in electrostatic interactions, stabilizing substrates or transition states during enzyme-catalyzed reactions [5]. This interaction is particularly important in enzymes involved in processes including acetylation [6], methylation [7], ubiquitination [8] and sumoylation [9]. Therefore, numerous efforts are devoted to the development of lysine-targeting bioactive molecules including natural products and synthetic compounds [10–13]. Natural products are a rich source of bioactive compounds that target lysines. For example, manoalide irreversibly bind the lysine residues Lys6 and Lys79 of phospholipase A2 (PLA2) enzyme [14], and Wortmannin covalently irreversibly modifies the active site lysine residue Lys833 of phosphoinositide 3-kinase (PI3K) [15]. More examples include curcumin [16], resveratrol, quercetin [17], allicin, berberine [18] and other plant-derived compounds that possess lysine-targeting properties and exhibit potential therapeutic effects. Covalent reversible inhibitors targeting lysine residues have also been developed. Akcay *et al.* first developed a covalent reversible inhibitor targeting myeloid cell leukemia 1 (Mcl-1) protein, which can covalently modify Lys234 through an aryl boronic acid carbonyl warhead. PPY-A developed by Yao *et al.* also covalently and reversibly targeted the Lys271 of BCR-ABL [19]. As lysine residue is important in various biological functions and processes, it is crucial to understand the proteome-wide selectivity of these lysine-targeting bioactive molecules.

Among various chemical proteomics approaches to identification of protein target, activity-based protein profiling (ABPP) is now most frequently used to identify the protein targets of

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bioactive molecules [20,21]. Traditional ABPP technique relies on a protein-reactive probe to fulfill the enrichment and identification of protein targets. These protein-reactive probes can be divided into two classes. One class is derived from the bioactive compounds themselves [22]. For example, the artemisinin-derived probes developed by our group and other groups showed that artemisinin could covalently modify a large number of proteins in malaria parasites [23] and cancer cells [24]. The other class is by virtue of universal tool probes that could modify specific amino acid residues, such as cysteine [25] and lysine [26]. Pretreatment of the whole proteome with the intact bioactive molecules will occupy their intended binding sites where the tool probes do not touch. This discrepancy could be resolved by the quantitative proteomics. These tool probes are therefore valuable because no structural modification is required for the studied bioactive molecules. However, the majority of the reported probes are designed for cysteine residues, and only a limited number of probes are specifically developed for lysine residues [27–29]. In addition, most of tool probes are tagged with an alkyne handle that undergoes click chemistry during the pull-down process (Fig. 1A). However, the click chemistry could induce nonspecific background proteins, which will significantly affect the accuracy of quantitative proteomics [30,31]. Herein, we report a pair of integrated lysine-reactive tool probes consisting of lysine-reactive warhead, a desthiobiotin moiety and isotopicable linker. Desthiobiotin's strong yet reversible binding to streptavidin allows consistent proteolytic digestion steps and easy peptide elution under acidic conditions with acetonitrile. By using desthiobiotin as an affinity handle, we could release and collect probe-modified peptides with acidic condition, which avoids not only click chemistry but also cleavable linkers. When used in a competitive manner, this pair of tool probes can be employed to identify the target proteins of bioactive molecules that react with lysine residues in a covalent reversible manner.

We designed and named the click-free isoTOP-ABPP probe DBGGR that contains a warhead of activated ester, a diglycine linker and a desthiobiotin moiety (Fig. 1B). Activated esters display good solubility and preferred reactivity with amines [32]. They could also form stable adducts with proteinaceous lysines, which can be characterized by tandem-MS methods. The designed activated esters included tetrafluorophenyl ester, pentafluorophenol ester, and *N*-hydroxysuccinimide (NHS) ester. Desthiobiotin can bind strongly and reversibly to streptavidin, which enables the peptides to be easily eluted by acidic mixture solvent of acetonitrile and water. The reversible binding between desthiobiotin and avidin beads avoids the introduction of complexed cleavable linkers [33–36]. We used two isotopically differentiated glycine residues with a total mass difference of 6 Da, resulting in a design of heavy and light lysine-targeting tool probes (Fig. 1B). This pair of isotopic probes could enable a simplified and efficient workflow to identify the protein target of lysine-targeting bioactive molecules (Fig. 1C).

We synthesized the designed probes according to the synthetic route (Fig. 2A). Briefly, the amino group and carboxyl group of glycine was protected by the Cbz group and the *tert*–butyl group to give intermediate **4**, which was treated with $H_2$ to generate the glycine butyl ester. The glycine butyl ester was next coupled with Cbz-protected Gly and then treated with $H_2$ to give the intermediate **8**. Biotin was desulfurized by Raney nickel in an aqueous phase, followed by pH adjustment and recrystallization to produce desulfurized biotin. This desulfurized biotin was then coupled with NHS to form desulfurized biotin NHS ester. The next step involves the coupling of desulfurized biotin NHS ester with intermediate **8**, followed by treatment of trifluoroacetic acid to yield the intermediate **13**. Finally, a coupling reaction between **13** and NHS, pentafluorophenol, or tetrafluorophenol in presence of EDCI yielded three activated ester probes, DBGGNHS, DBGGTFP and DBGGPFP. In the initial screen of aqueous stability, we found that tetrafluorophenyl and pentafluorophenol esters are more stable than NHS ester (Fig. 2B), which is consistent with previous report [27,37]. We next compared proteomic reactivity of DBGGTFP and DBGGPFP in the whole proteome of U87MG. To do so, we employed the pull down *via* avidin beads to separate the labeled proteins and then resolved these proteins in sodium dodecyl sulphate-polyacrylamide gel

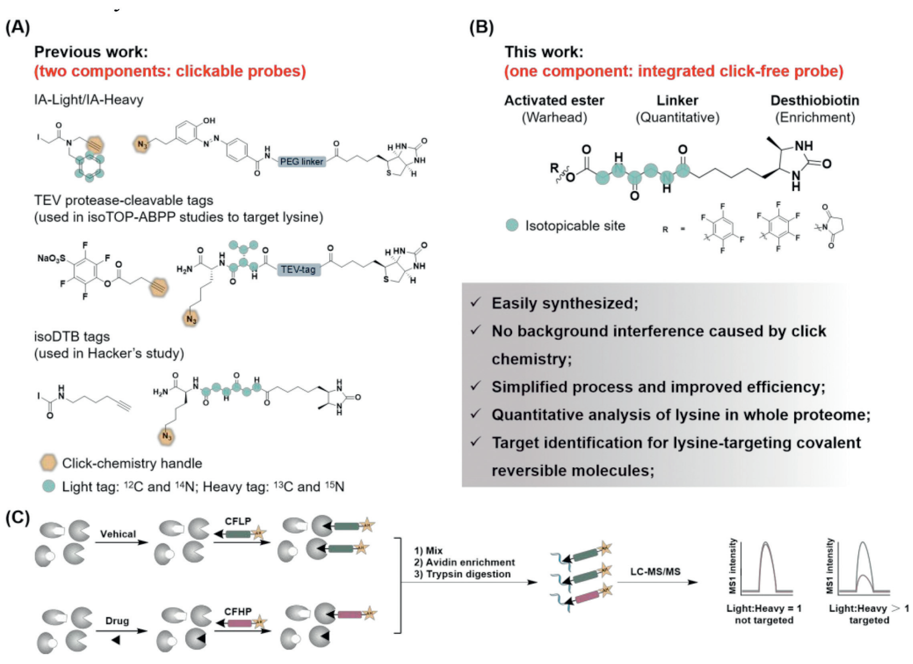
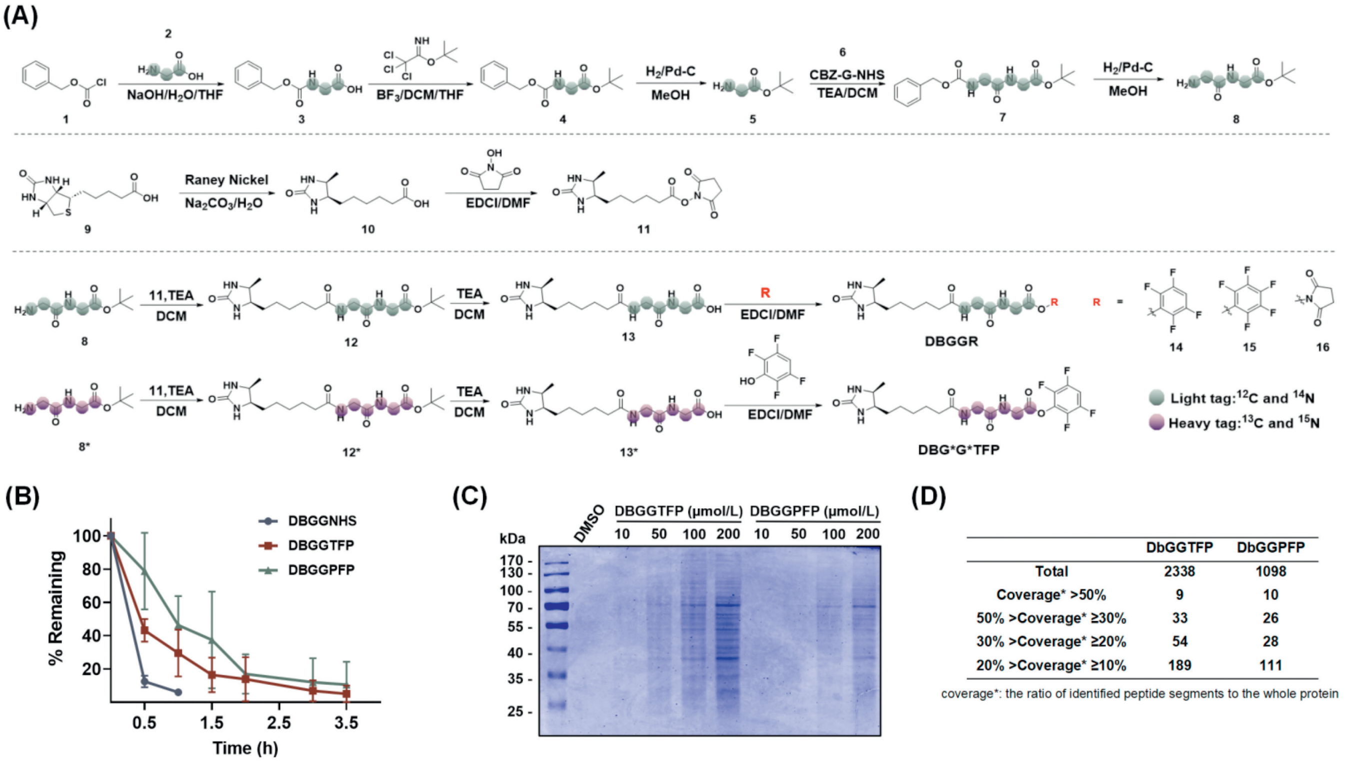


**Fig. 1.** Click-free probes and the corresponding workflow used in this study for lysine profiling. (A) Structures of the previously-utilized clickable ABPP probes for isoTOP-ABPP studies. (B) The general structure of click-free DBGGR in this work. (C) The workflow for target identification of lysine-targeting bioactive molecules *via* DBGGR. AH, affinity handle; CFLP, click-free light probe; CFHP, click-free heavy probe.



| | DbGGTFP | DbGGPFP |
|---|---|---|
| Total | 2338 | 1098 |
| Coverage* >50% | 9 | 10 |
| 50% >Coverage* ≥30% | 33 | 26 |
| 30% >Coverage* ≥20% | 54 | 28 |
| 20% >Coverage* ≥10% | 189 | 111 |

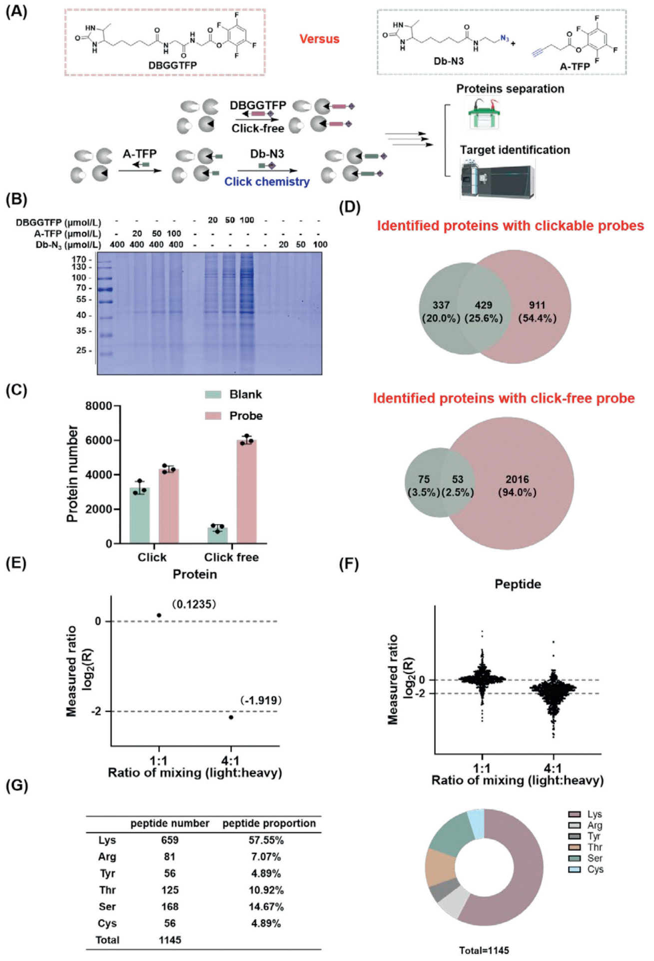
coverage*: the ratio of identified peptide segments to the whole protein

**Fig. 2.** Synthesis and bioactivity evaluation of isotopic probes. (A) Synthesis of DBGGR and DBG*G*TFP. (B) The hydrolytic stability of DBGGR in PBS buffer. (C) In-gel based comparison of protein-targeting capability between DBGGTFP and DBGGPFP. (D) LC/MS-MS based comparison of protein-targeting capability between DBGGTFP and DBGGPFP.

electrophoresis (SDS-PAGE) gel and stained them with Coomassie blue. As shown in Fig. 2C, both DBGGTFP and DBGGPFP could label multiple proteins in a concentration-dependent manner, and DBGGTFP had higher labelling ability than DBGGPFP. To further validate the gel-based result, we performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of the proteins pulled down by two probes. The pull down with DBGGPFP and DBGGTFP identified 1098 and 2338 proteins, respectively (Fig. 2D). The lousy performance of DBGGPFP was probably because DBGPFP has slightly lower solubility compared to DBGGTFP (Fig. S1 in Supporting information). We therefore selected DBGGTFP as the probe for the following proteomic study. Accordingly, we proceeded to synthesize heavy isotope-labeled probe, DBG*G*TFP. The synthesis started with glycine with two $^{13}$C atoms and one $^{15}$N atom as the starting material, which resulted in a 6-Da difference between DBGGTFP and DBG*G*TFP. DBG*G*TFP was successfully synthesized with the same route as the light-labeled probe. The structure of DBG*G*TFP was verified correct *via* nuclear magnetic resonance (NMR) (appendix) and high resolution mass spectrometry (HRMS) (Fig. S2 in Supporting information).

After selection of DBGGTFP as the tool probe, we next compared DBGGTFP in our click-free assay with the corresponding clickable probe (A-TFP) in terms of protein capturing ability in human cell proteomes. To do so, we synthesized the clickable probe A-TFP and its click partner Db-$N_3$ containing a desthiobiotin and azide handle (Fig. 3A, Schemes S1 and S2 in Supporting information). For the click group, the cell lysate (2 mg/mL) was first incubated with different concentrations of A-TFP (20–100 μmol/L), followed by conjugation with Db-$N_3$ *via* CuAAC. For the click-free group, the cell lysate (2 mg/mL) was directly incubated with different concentrations of DBGGTFP (20–100 μmol/L). For the control group, we treated the cell lysate (2 mg/mL) with DMSO, followed by click chemistry with DB-$N_3$ (Fig. 3B). The proteomes in the three groups were then incubated with an equal amount of avidin beads. The labeled proteins were enriched and then released from the beads by heating at 90 °C for 5 min. Next, the samples were denatured with SDS loading buffer, followed by heating at 95 °C for 10 min. The acquired proteins were next resolved by SDS-PAGE gel and stained with Coomassie blue. As same as our previous observation [38], the result indicated that DBGGTFP group without click chemistry displayed higher levels of labeling than A-TFP group (Fig. 3B). We also observed that click reagents could introduce some background proteins while the background of click-free group is much cleaner. To further compare the clickable probe with click-free probe, we examined the number of proteins pulled down by these probes with LC-MS/MS in each sample. For the click-free group, the cell lysate (2 mg/mL) was incubated with DBGGTFP (0 or 100 μmol/L). The treated proteome was enriched and separated by avidin beads, followed by direct on-beads digestion and protein identification *via* LC-MS/MS. For the click group, the lysate was treated with A-TFP (0 or 100 μmol/L). The treated proteome was then first clicked with DB-$N_3$ (400 μmol/L), followed by enrichment, digestion and identification. As shown in Fig. 3C, in three independent pull-down experiments conducted with 0 μmol/L and 100 μmol/L DBGGTFP, an average of 922 proteins and 6019 proteins were identified, respectively. While the pull down with 0 μmol/L and 100 μmol/L A-TFP identified 3242 proteins and 4333 proteins, respectively. The ratio of overlapped proteins in A-TFP (0 μmol/L) and A-TFP (100 μmol/L) group was 25.6%, while in DBGGTFP (0 μmol/L) and DBGGTFP (100 μmol/L) group was only 2.5% (Fig. 3D). This result is in line with the gel-based result, and indicates that the click chemistry in the pull-down process could induce a large number of non-specific background proteins. This result also demonstrated that our integrated probe DBGGTFP could efficiently capture the protein targets with minimal background interference.

We next examined the ability of light and heavy DBGGTFP to accurately quantify proteins. First, we treated two identical sam-



| | peptide number | peptide proportion |
|---|---|---|
| Lys | 659 | 57.55% |
| Arg | 81 | 7.07% |
| Tyr | 56 | 4.89% |
| Thr | 125 | 10.92% |
| Ser | 168 | 14.67% |
| Cys | 56 | 4.89% |
| Total | 1145 | |

**Fig. 3.** The click-free probe DBGGTFP is better than its corresponding clickable probe in the process of target identification and the evaluation of quantification accuracy of the isotopic probes. (A) The chemical structure of clickable probe (A-TFP, top right) *versus* click-free probe (DBGGTFP, top left) and the workflow of target identification using the click-free probe DBGGTFP and the clickable probe A-TFP. (B) The concentration-dependent protein labeling of A-TFP and DBGGTFP in U87MG cell lysates. The labeled proteins were stained with Coomassie blue. (C) The average number of identified proteins in each pull-down process with or without A-TFP and DBGGTFP, each point represents an independent experiment. Data are presented as mean ± standard deviation (SD) ($n = 3$). (D) Overlap of identified proteins between the control group (light green) and the probe group (light purple), by using clickable probes or the click-free probe. The identified proteins in three independent experiments were intersected. (E) $R$ of quantified protein in the BSA pure protein experiments, in which the light and heavy labeled samples were separately reacted with 1 mmol/L DBGGTFP and 1 mmol/L DBG*G*TFP and mixed at the indicated ratios. Dashed grey lines indicate expected $\log_2(R)$ values of 0 for the 1:1 mixture and 2 for the 1:4 mixture. (F) $R$ of quantified lysines in U87MG proteome. The procedure was same as that in Fig. 3E, except that the pure protein was changed into cell lysate. (G) Distribution of probe-modified peptides in U87MG lysis proteome assessed by click-free integrated probe DBGGTFP.

ples of bovine serum albumin (BSA) with 100 μmol/L DBGGTFP or DBG*G*TFP. The treated BSA samples were combined in a ratio of 1:1 or 1:4. Subsequently, the probe-modified BSA was enriched on streptavidin beads and digested with trypsin. The probe-modified peptides were eluted with 50% acetonitrile/$H_2O$ with 0.1% formic acid (FA), and analyzed with tandem mass. The proteomic data was finally resolved by Msfragger software to generate the detected ratio of the light probe-labeled BSA and heavy probe-labeled BSA. As shown in Fig. 3E, the detected ratios were near the expected value, which demonstrated that our newly-developed probes could accurately quantify proteins. To further demonstrate that our probes are applicable to quantify the proteins in a proteomic context, we next treated two identical samples of the lysate of U87MG with 100 μmol/L light or heavy DBGGTFP. The subsequent operations were as same as the experiment with pure BSA. As shown in Fig. 3F, the detected ratios were tightly clustered around the expected values. Therefore, the light and heavy DBGGTFP probes could reliably perform the quantification of proteins in the whole cell proteome.

Previous studies have shown that activated esters can react with lysine and other nucleophilic amino acids including serines, threonines and tyrosines [27]. We next profile the binding sites of DBGGTFP. Over half of these sites (57.55%) were lysines, being consistent with the known preference of activated esters to amines (Fig. 3G). The result thereby indicated that DBGGTFP shows high reactivity and good selectivity for lysine residues in the human proteome.

We next applied our new approach to identifying the protein targets of Gossypol. We selected Gossypol as the studied bioactive molecule because of two reasons. First, Gossypol has the structure of hydroxybenzaldehyde that could covalently form a Schiff-base with lysine [39,40]. Second, the reversibility of covalent reaction between Gossypol and lysine has not been studied so far. We subsequently employed high performance liquid chromatography (HPLC) and NMR to study the reaction. As shown in Figs. 4A and B, the amino group in the side chain of lysine could efficiently react with and consume up the Gossypol to generate the single adduct and double adduct. The formation of the Schiff-base was also confirmed by the appearance of hydrogen peak at the chemical shift of 9.7 ppm (Fig. S3 in Supporting information). When the reaction mixture was diluted, the amount of double adduct gradually decreased and the Gossypol appeared and increased in a time-dependent manner (Figs. 4C and D). The NMR spectra of diluted reaction mixture showed that the area of hydrogen peak at the chemical shift of 9.7 gradually decreased (Fig. S3), indicating the formed Schiff base gradually disappeared. These two experiments clearly demonstrated that the covalent reaction between Gossypol and lysine is reversible. This result is consistent with previous reports that short-term use of Gossypol use can restore reproductive vitality after discontinuation [41,42] and majority of users with long-term use experienced reversible contraceptive effect [43,44]. It is thereby quite challenging to employ the pull-down based approaches to identify the protein targets of Gossypol as it may get off the beads during the enrichment. However, our new pair of isotope-labeled probes could in principle differentiate and identify the targeting lysines and their corresponding proteins.

To identify the protein targets of Gossypol, we preincubated U87MG cell lysate with vehicle or Gossypol, followed by competitive labeling with isotopically light or heavy DBGGTFP. Then the two batches of proteome were combined and probe-modified proteins were enriched and digested. Probe-modified peptides were eluted using acidic solvent and analyzed by LC-MS/MS (Fig. 4E). The intensity ratios ($R$) of probe-modified peptides with light tag to that with heavy tag were quantified. For those peptides whose lysines are not bound by Gossypol, their corresponding ratios between the heavy and light channel are close to 1 ($R = 1$). For those peptides whose lysines are bound by Gossypol, the ratios are bigger than 1 ($R \geq 1$). Because the modified peptide is directly detected, this technology cannot only determine the target protein, but also determine the exact interaction site. In addition, we also treated the cell lysates with Gossypol at room temperature for 1 h, at concentrations ranging from 20 μmol/L to 100 μmol/L. For further analysis, we focused on the proteins that were significantly engaged ($\log_2(R) > 2$, $P < 0.05$) (Fig. 4F). As shown in Fig. 4F, higher concentration of Gossypol was used, more proteins will meet the above standard. We also draw heat maps indicating that the labeling between the Gossypol and targeted protein

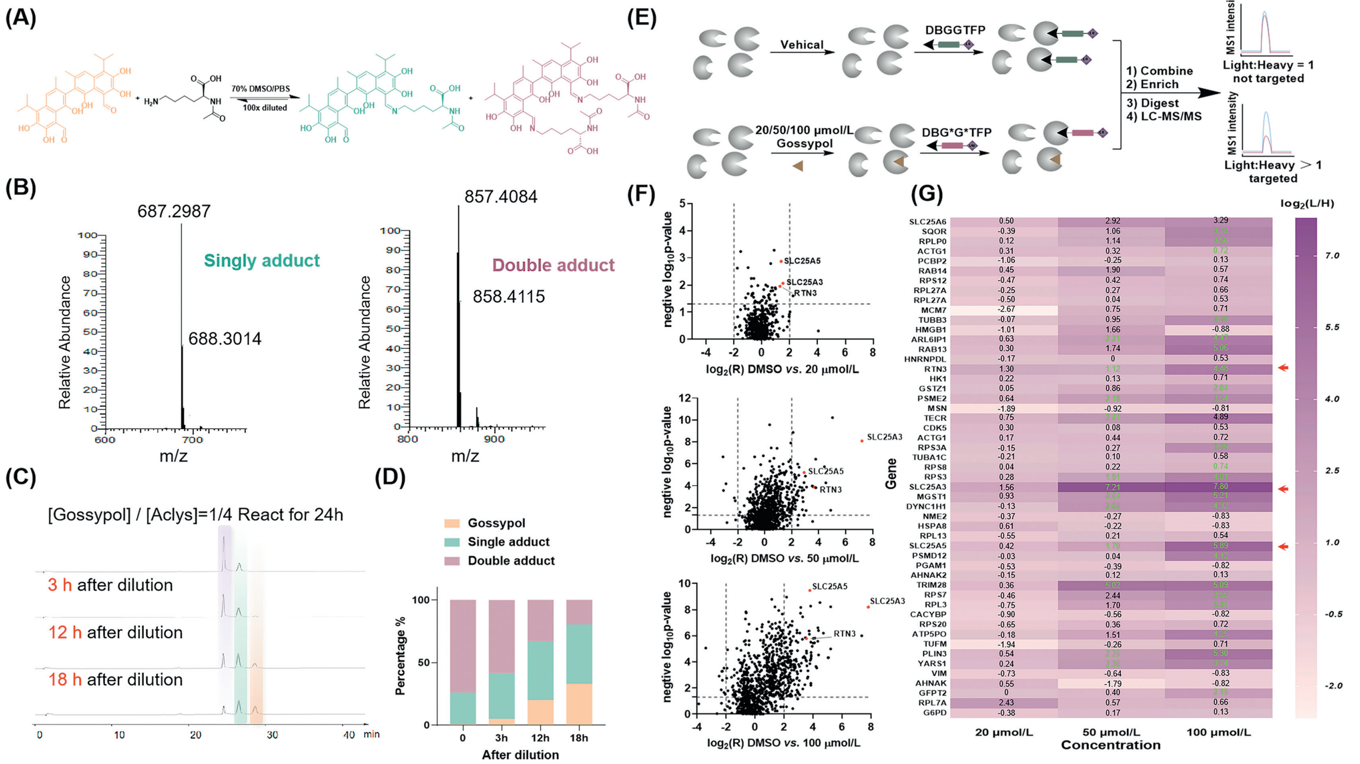


**Fig. 4.** The binding between Gossypol and lysine is covalent reversible and identification of protein targets of Gossypol *via* DBGGTFP tags. (A) The reaction equations for Gossypol and acetyl-lysine. (B) HRMS spectra of single adduct and double adduct. (C) HPLC tests were performed on the reaction mixture of Gossypol and acetyl-lysine after a 100-fold dilution. (D) The ratio of Gossypol, single adduct and double adduct in the reaction mixture after a 100-fold dilution. (E) The workflow of DBGGTFP-based ABPP to identify protein targets of Gossypol. (F) Volcano plots of the Click free DBGGTFP-based ABPP experiments that show the median $\log_2(R)$ of the ratio between the light (DMSO-treated) and heavy (Gossypol-treated) channels and the $\log_{10}(P)$ of the statistical significance in a one-sample *t*-test for all quantified lysines for Gossypol at 20, 50 and 100 μmol/L. (G) Concentration dependence of the degree of competition determined using Click-free DBGGTFP-based ABPP for lysines, the red arrow indicates proteins exhibiting significant concentration dependence, which were subsequently validated *via* WB analysis.

was in a concentration-dependent manner. In total, 50 protein targets showed clear concentration-dependent manners (Fig. 4G, Tables S1–S3 in Supporting information).

We next employed Western blot (WB) to verify the proteomics result. Three proteins were selected, solute carrier family 25 member 3 (SLC25A3), ADP/ATP translocase 2 (SLC25A5) and reticulon-3 (RTN3), due to their strong concentration-dependent relationship between the ratio *R* and Gossypol concentration. To do so, U87MG cell lysate was incubated with different concentrations of Gossypol for 1 h and then treated with 100 μmol/L DBGGTFP for 1 h. Subsequently, 200 μL of reaction solution was taken out as the input. Remaining solution was then incubated with avidin beads, and the labeled proteins were enriched and then released from the beads by boiling the beads as output. The acquired input and output proteins were next resolved by SDS-PAGE gel and imaged with WB as well as Coomassie blue (Figs. 5A and B), the un-cropped WB images were shown in Figs. S4–S6 (Supporting information). To our surprise, the intensity of WB bands showed a concentration-dependent decrease with the increase of Gossypol concentration not only in the output group, but also in the input group (Fig. 5A). It is easy to interpret the result in the output group as Gossypol competed with the probe for binding to these three target proteins (Figs. 5C–E). But it took us lots of efforts to fully understand the phenomenon observed in the input group. We speculate that the binding of Gossypol to the specific lysine residue may stop the antibody from binding its target proteins. To verify this speculation, we treated 1 mg/mL or 2 mg/mL U87MG cell lysate with 100 μmol/L Gossypol for 1 h, followed by protein precipitation and WB experiment (Fig. 5F). Moreover, when the concentration of Gossypol is held constant, a higher proportion of proteins is blocked

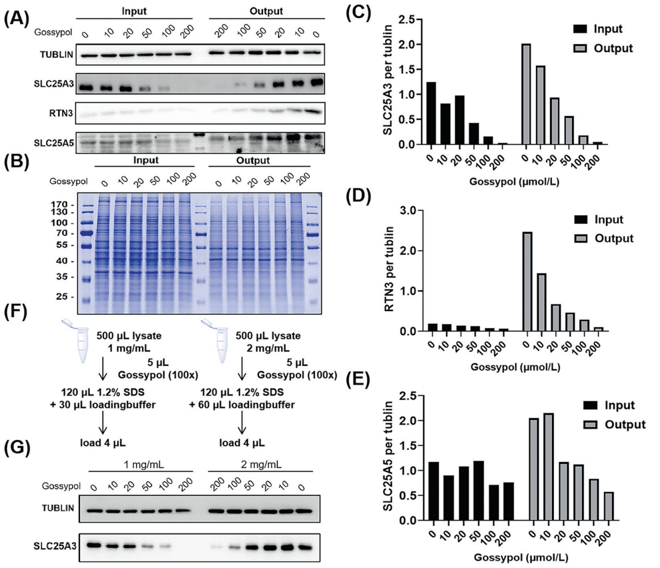


**Fig. 5.** The orthogonal validation of proteomics results. (A) WB detection of selected proteins labeled by Gossypol (0/10/20/50/100/200 μmol/L) for 1 h and then incubated with DBGGTFP (100 μmol/L) for 1 h ('input'), pulled down by avidin beads and eluted by boiling the beads ('output'). (B) Coomassie brilliant blue staining of the input lysate in (A). (C) The quantitation analysis of WB result of SLC25A3. (D) The quantitation analysis of WB result of SLC25A5. (E) The quantitation analysis of WB result of RTN3. (F) The workflow of WB samples preparation to confirm Gossypol blocking antibody binding by using two protein concentrations. (G) WB detection of SLC25A3 in two concentrations of U87MG lysate labeled by Gossypol.

at lower protein concentrations (Fig. 5G). The results clearly indicated that the Gossypol could prevent the antibody from binding SLC25A3 protein. The concentration dependence of the input group is presumed to be due to the binding of Gossypol to the protein which blocked the antibody binding site, since that Gossypol has a large size. In addition, the anti-RTN3 antibody recognizes the sequence of "QIDHYVGIARDQTKSIVEKIQAKLPGIAKKKAE" (https://www.uniprot.org/uniprotkb/O95197) (Fig. S7 in Supporting information), while our previous result in Fig. 4F demonstrated that Gossypol could modify this peptide ($R = 3.65$) (Fig. S8A and B in Supporting information). These WB and mass results indicated that Gossypol could indeed stop its modified proteins being recognized by their corresponding antibodies (Fig. S8C in Supporting information). Nevertheless, these results undoubtedly indicated that our isotopic probes could efficiently and correctly identify the protein target of covalent reversible bioactive molecules.

Covalent inhibition could render the corresponding inhibitor with longer and stronger pharmacological effect compared to the noncovalent inhibition. But these proteins modified by covalent inhibitors may cause the immune-related side effect. Along this line, the covalent reversible inhibitors could be safer than the covalent irreversible inhibitors [45]. The reversibility of covalent inhibitors mainly has two interpretations. First, the reversibility refers to a reverse biological process, in which the covalent modification could be removed by certain enzymes. For example, Aspirin covalently modifies hundreds of protein targets at various residues including lysine, serine and threonine [46]. But this acylation could be erased by deacetylases so that the aspirin-induced acylation is biologically reversible. The other interpretation of covalent reversible inhibitors is that the resulting modification is chemically reversible. The covalent modification could reverse automatically and no enzyme is needed in this process. The covalent reversible modification demonstrated in this manuscript refers to the second interpretation. Covalent reversible inhibitors can be obtained from organic synthesis [47] and natural product isolation [48]. Most of reported covalent reversible inhibitors are generated from the organic synthesis. It seems that the natural product-derived covalent reversible inhibitors are less explored. We proved that Gossypol has the covalent reversible function, which may open up a new area where other compounds with hydroxybenzaldehyde structure may also have the covalent reversible function. A drawback of Gossypol is that its reversibility is good and slow. Further structural modification of Gossypol to improve its capacity of reversibility deserves a deep examination.

The identification of protein targets is crucial to understand the mode of action and proteomic selectivity of bioactive molecules. Among the various approaches of target identification, the pull-down based approach is widely employed. For covalent irreversible inhibitors, it is a challenge to identify their protein targets *via* the pull-down based approach as they may get off the beads during the process of target separation and enrichment. As such, the competitive ABPP is widely and successfully used to identify the protein target of covalent reversible inhibitors [49–51]. This approach is highly dependent on a versatile tool probe that could covalently modify specific kinds of residues of a large number of proteins. Lots of these probes have been developed, for example, IA-Alkyne [25], IA-light/IA-heavy [52], IsoDTB [34], STP alkyne [26], to name just a few. A common property of these probes is that they all have an alkyne handle. They need to react with the azide-linked biotin or dethiobiotin to finish the identification of target proteins. According to the previous reports, the clickable probe works better than the integrated probe in terms of fluorescent gel labeling experiment although it needs two steps [52,53]. However, these previous reports only refer to the protein labeling with a fluorescent dye. The fluorescent dye (*i.e.* rhodamine) has such a big size that the integrated probe may render the warhead unable to react with its target site because of the steric hindrance. But the fluorescent labeling in a typical ABPP assay is usually to check and optimize the labeling condition. Regarding the pull-down process, the fluorescent dye is usually changed to biotin or desthiobiotin. However, little study has been carried out to compare efficiencies of target identification between the clickable probe and integrated probe in the pull-down process. Biotin or desthiobiotin has much smaller size than the fluorescent dye. The steric hindrance of desthiobin almost disappears as the proteins identified by the integrated probe include nearly all the proteins in the clickable group. The clickable probe needs two steps that compromised the efficiency of target identification. In addition, click reactions also increase the background signal [30,31]. Additionally, the integrated probes offer the advantage of convenience. For example, the workflow is straightforward, ensuring that future users can avoid potential technical challenges and save time in their experimental procedures. Certainly, the larger size of integrated probes may lead to reduced cellular penetration. Additionally, the cost-effective choice of a relatively short isotopically-labeled linker can result in undesirable steric hindrance. These aspects merit further refinement and optimization of integrated linkers. Taking these considerations into account, the integrated probe is more suitable than the clickable version as a tool probe to identify the protein target of lysine-modifying bioactive small molecules in a covalent reversible manner.

In summary, we have developed a pair of proteomic-useful integrated probes, light and heavy-DBGGTFP. These two probes have the same chemical structure and a 6-Da difference in terms of molecule weight. We proved that the integrated probe works much better than the clickable probe in the pull-down process of target identification. We also successfully applied this pair of probes in the quantitative analysis of reactive lysine in a context of whole proteome. In addition, we for the first time demonstrated that the Gossypol could react with lysine in a covalent reversible manner. The protein targets of Gossypol could be accurately identified by using our new integrated probes. The development of integrated probe and discovery of the new covalent reversible warhead will definitely promote the development of lysine-targeting covalent reversible inhibitors. Meanwhile, given that the probes react with lysines which are crucial modification sites in proteins, we anticipate broader applications in the investigation of post-translational modifications (PTMs), protein-metabolite interactions, and other related studies. It offers a versatile tool for exploring diverse aspects of cellular processes, enhancing our ability to decipher intricate biological mechanisms.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

**Xingyu Ma:** Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Yi-Xin Chen:** Writing – review & editing. **Zi Ye:** Software. **Chong-Jing Zhang:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

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## Supplementary materials

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