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Anticancer pyrroloquinazoline LBL1 targets nuclear lamins

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Abstract

Target identification of bioactive compounds is critical for understanding their mechanism of action. We previously discovered a novel pyrroloquinazoline compound **LBL1** with significant anticancer activity. However, its molecular targets remain to be established. Herein, we developed a clickable photoaffinity probe based on **LBL1**. By extensive chemical, biochemical and cellular studies with this probe and **LBL1**, we found that **LBL1** targets nuclear lamins, which are type V intermediate filament (IF) proteins. Further studies showed that **LBL1** binds to the coiled-coil domain of lamin A. These results revealed that IF proteins can also be targeted with appropriate small molecules besides two other cytoskeletal proteins actin filaments and microtubules, providing a novel avenue to investigate lamin biology and a novel strategy to develop distinct anticancer therapies.

Graphical Abstract

INTRODUCTION

Identification of small molecule ligands to various biological macromolecules is critical for drug discovery to provide hit compounds or chemical probes for target biology discovery. Various sources including natural products and screening compound collections can contribute to the identification of initial hits.^{1–2} Despite the rapid advances in recombinant technology and genomic sciences, phenotypic screening still plays a pivotal role in hit discovery in today's drug discovery activities especially in disease areas with complex etiologies.³ In fact, an analysis of FDA-approved first-in-class drugs between 1999 and 2008 showed that while only 34% of the drugs were originated from target-based strategies, phenotypic screens contributed to up to 56% of the approved drugs.³

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Critical to a phenotypic screen is the availability of high-quality library compounds. Along this line, derivatives from privileged scaffolds, initially coined by Evans,⁴ are fruitful grounds for novel bioactive compounds and many such focused libraries have been prepared.^{5–6} We previously developed a unique suite of chemical methods to prepare a focused library of privileged acylpyrroloquinazolines.⁷ From this library of unique compounds, we discovered **LBL1** with significant anticancer activities (Figures 1A and S1).⁷ While we excluded a few potential targets,⁷ **LBL1**'s direct molecular target(s) remain unknown. In this article, we employed an unbiased chemoproteomics strategy⁸ with a clickable photocrosslinking probe **LBL1-P** to identify **LBL1**'s direct binding targets. Using this analysis as well as additional biochemical and cellular supporting studies, we show that **LBL1** targets nuclear lamins, which are type V intermediate filament (IF) proteins and have not been successfully targeted by small molecules.⁹

RESULTS AND DISCUSSION

We envisioned that **LBL1-P** would covalently crosslink with its direct binding target(s) in living cells upon UV-irradiation of **LBL1-P**-treated cells (Figure 1B). Then the covalently crosslinked target(s) could be clicked⁸ with an appropriately-tagged azide for further downstream applications (Figure 1B). The photocrosslinking probe **LBL1-P** was designed after investigating the preliminary structure-activity relationships of **LBL1** in inhibiting breast cancer cell growth in MDA-MB-468 and MDA-MB-231 cells (Table 1). Placing a clickable propargyl group at *N*-7 of **LBL1** resulted in compound **1**, which showed only slightly decreased activity compared to **LBL1**. We considered both benzophenone (**2**) and trifluoromethyldiazirine (**3**) as potential photoaffinity labels. It was determined that **3** had more potent activity than **2** (Table 1). Based on these results, we designed **LBL1-P** (Figure 1A). **LBL1-P** was synthesized as shown in Scheme 1 starting from previously made parent compound **4**.⁷ Propargylation at *N*-7 of **4** with NaH and propargyl bromide generated **5**, whose *N*-1 amine was temporarily converted into a –OH group. Amide formation of between **6** and NHS ester **8** provided **9**. Final regeneration of *N*-1 amine⁷ was achieved by BOP/DBU followed by a S_NAr reaction with NH₃ to yield desired compound **LBL1-P**. The synthetic details for compounds **1–3** are provided in the Supporting Information. Gratifyingly, **LBL1-P** retained growth inhibitory activities in both of the cell lines albeit with a slightly reduced potency compared to **LBL1** (Table 1). It is interesting to note that **LBL1-P** was slightly more potent than compound **3** in MDA-MB-231 cells, but the opposite was true in MDA-MB-468 cells. The reason for this difference remains to be determined.

To investigate if **LBL1-P** could label its target(s), MDA-MB-468 cells were treated with **LBL1-P** in the absence or presence of **LBL1** as a competitor. After UV (365 nm) irradiation, the cell lysates were clicked with a biotin-N₃.¹⁰ The crude cell lysates were then separated on a SDS-PAGE gel and the biotinylated proteins were directly detected by horseradish peroxidase (HRP)-conjugated streptavidin blotting. The results in Figure 2A showed that three prominent bands migrated between 60 and 75 kDa were specifically labeled by **LBL1-P**. Importantly, this labeling was reduced with excess **LBL1**. Labeling of these three bands was fairly specific and no other major bands were labeled even at 10 μM of **LBL1-P**, which is well above its GI₅₀ (Figure 2A and Table 1).

To identify the proteins that were labeled by **LBL1-P**, we enriched the photocrosslinked and biotin- N_3 clicked cell lysates with streptavidin beads for mass spectrometry-based protein identification. The relative abundance of the enriched proteins was quantified by label-free spectra counting. This analysis revealed nuclear lamin A (LA), lamin B1 (LB1), lamin B2 (LB2) and lamin C (LC) as the putative targets of **LBL1** (Figure 2B and Table S1). Lamins are type V intermediate filament (IF) proteins and are the major component of nuclear lamina, a meshwork of proteins that lie underneath the inner nuclear membrane.^{11–12} In mammals, there are three lamin genes (*LA*, *LB1* and *LB2*) encoding four major proteins (LA, LB1, LB2 and LC). LA and LC are alternative splicing products of *LA* gene.^{13–14} All the lamin proteins are highly homologous in sequence and predicted structure and share the same domain organization. They contain a long central, α -helical coiled-coil rod domain¹⁵, which is flanked by a non- α -helical *N*-terminal head and a *C*-terminal immunoglobulin (Ig)-like tail domain.¹⁴ Lamins are the major scaffold proteins to maintain the mechanical stability of the nucleus and have been considered as undruggable proteins. Therefore, our identification of lamins as the potential targets of **LBL1** was unanticipated.

To determine the localization of the labeled proteins by **LBL1-P**, we separated the cytosolic fraction from the nuclear fraction from the cells treated with **LBL1-P**. Each fraction was individually clicked with a rhodamine- N_3 for in-gel fluorescence imaging.¹⁰ The labeled signals were found to be exclusively localized in the nuclear fraction (Figure 2C) and could be competed by **LBL1**, which is consistent with the finding that lamins are the targets of **LBL1**. The purity of cytosolic and nuclear fractions was confirmed by the presence and absence of cytosolic GAPDH and nuclear histone 3 (H3) in individual fractions through western blotting (Figure 2C).

We used western blot as an independent method to validate that lamins were truly and specifically pulled down by **LBL1-P**. Therefore, the photocrosslinked and biotin- N_3 -clicked cell lysates were pulled down with streptavidin beads and probed with anti-LA/C, LB1 and LB2. The data in Figure 3A showed that LA/C and LB1 were pulled down and again the efficiency was decreased when **LBL1** was included as a competitor. The labeling efficiency was dose-dependently inhibited by **LBL1** (Figure S2). LB2 was not detected in the pulldown (Figure 3A). To distinguish if lack of LB2 in the pulldown was due to lower binding affinity or inferior antibody performance, we ectopically expressed FLAG-tagged LA, LB1 and LB2 in HEK293T cells and performed the same photocrosslinking and streptavidin pulldown as shown in Figure 3A. Then the proteins being pulled down were detected by anti-FLAG antibody. It was found that all three of them could be pulled down and detected by anti-FLAG antibody (Figure 3B) yet LB2 clearly presented significantly lower labeling efficiency in comparison to LA and LB1, suggesting that **LBL1-P** binds less avidly with LB2. Upon inspection of the sequence alignment among LA, LB1 and LB2, we found that the *N*-terminal sequence of LB2 is longer than that of LA or LB1 (Figure S3), suggesting that this portion of sequence might be important for binding. Lamins are abundant nuclear proteins. To exclude that **LBL1-P** simply pulled down abundant cellular proteins, we investigated other highly abundant cellular proteins including Hsp90 and GAPDH from the enrichment and we did not detect either of these (Figure 3A). Others have attempted to inventorize the nonspecific proteins that could be enriched using clickable photoaffinity

probes and indeed lamins were not found to be present in the background inventory using a variety of photoreactive groups.¹⁶ These results suggest that **LBL1** binds to lamins. This is the first small molecule ligand known to bind lamins and thus the name of **LBL1** for lamin-binding ligand 1.

To further confirm that **LBL1** could target LA in cells, we investigated the localization of **LBL1-P** in MDA-MB-231 cells by in-cell click reaction with a rhodamine-N₃ after photocrosslinking. The cells were also stained with anti-LA. When the cells were treated with **LBL1-P** only, a strong rhodamine signal was observed and this signal colocalized with LA (Figure 3C, top panel). To quantitatively evaluate the colocalization between **LBL1-P** and LA, we analyzed deconvoluted Z-stacks of cell images using Imaris® Coloc module and the colocalization coefficient was found to be nearly perfect at 0.92 ± 0.022 (Figure 3D). Similar to the SDS-PAGE analysis (Figures 2A and 3A), the signal labeled by **LBL1-P** was significantly decreased in the presence of excess **LBL1** (Figure 3C, middle panel), further supporting that **LBL1** targets LA in living cells. The specificity of the click reaction was confirmed by the lack of rhodamine signal from the cells that were not treated with **LBL1-P** (Figure 3C, bottom panel).

Full-length LA is very challenging to be biochemically characterized due to its tendency to aggregate in physiological buffers.¹⁷ To facilitate the biochemical characterization of the binding interaction between **LBL1** and LA, we employed photocrosslinking followed by click reaction with biotin-N₃ and streptavidin pulldown to identify the minimal fragment of LA to mediate the binding interaction. We generated a series of FLAG-tagged LA fragments that encompass different domains and expressed them in HEK293T cells. The transfected cells were then treated and processed as Figure 3B for anti-FLAG blotting. As expected, the full-length LA could be labeled by **LBL1-P** (Figures 4A and S4A). Surprisingly, we found that LA(1–427) consisting of the coiled-coil domain maintained robust labeling by **LBL1-P** while the Ig-like domain LA(428–664) did not (Figures 4A and S3A), although the Ig-like domain mediates interactions with many other proteins.^{18–19} LA(1–427) contains two coiled-coils. We further divided LA(1–427) into coil 1 [LA(1–218)] and coil 2 [LA(219–427)] to identify which coil might mediate the interaction with **LBL1**. However, neither coil 1 nor coil 2 alone was sufficient to crosslink with **LBL1-P** (Figures 4A–B), indicating that both coils are required for binding with **LBL1**. Then a series of shorter fragments containing both coils were prepared. While LA(1–387) mediated robust labeling, no labeling of LA(1–355) with **LBL1-P** was observed (Figures 4A–B), suggesting the C-terminal end of the non-helical coil is critical for binding. At the N-terminal end, a series of deletions of 10aa [i.e. LA(10–387), LA(20–387) and LA(30–387)] also presented deleterious effect on binding (Figures 4A and S4B). Minimal labeling of LA(30–387) was observed while no labeling was detected with LA(78–387). These results showed that the minimal fragment required for binding with **LBL1** was LA(1–387) consisting of the non-helical head domain (36aa), both coil 1 and 2, and ~30aa C-terminal to the end of the predicted coil 2 (Figure 4A). Together, these results suggest that there likely exists intermolecular interaction between N- and C-terminal ends in LA(1–387) fragment to maintain a functional assembly required for optimal binding with **LBL1**.

To assess the biochemical binding interaction between **LBL1** and LA(1–387), we purified LA(1–387) to homogeneity (Figure S5). As a negative control, LA(78–387) was also purified. We first employed limited proteolysis assay²⁰ of purified LA(1–387) by trypsin to assess its binding with **LBL1**. Binding of a small molecule to a protein will protect the protein from partial proteolysis.²⁰ Incubation of increasing concentrations of **LBL1** with LA(1–387) dose-dependently protected it from limited proteolysis by trypsin (Figure 4C), indicating direct binding interaction between **LBL1** and LA(1–387). As a negative control, partial proteolysis of LA(78–387) was not affected by **LBL1** (Figure 4D), indicating no direct binding with LA(78–387). We further exploited a thermal shift assay (TSA)²¹ to determine the binding between **LBL1** and LA(1–387). Ligands binding to a protein will increase its apparent melting point (T_m).^{21–23} As shown in Figure 4E, LA(1–387) showed two apparent thermal transitions in the absence of **LBL1** (T_{m1} ~40 °C, T_{m2} ~65 °C), likely reflecting unfolding of two different assembly states. Upon titrating **LBL1** into LA(1–387), T_{m2} was unchanged while T_{m1} was dose-dependently shifted to higher T_m (Figure 4F) with an apparent K_d = 5.11 μ M, which is in line with the results from the limited proteolysis assay (Figure 4C). Under the same conditions, no change in the T_m of glutathione S-transferase (GST) was observed (Figure S6). Collectively, these results illustrate that **LBL1** binds to LA(1–387) both *in vitro* and in living cells.

CONCLUSION

The current study details the identification of the direct molecular targets of anticancer agent **LBL1** with the assist of an unbiased chemoproteomics strategy. Unexpectedly, we found that **LBL1** targets nuclear lamins. The mammalian cytoskeleton is comprised of three protein classes, i.e. actin filaments or microfilaments (~8 nm in diameter), microtubules (~25 nm in diameter) and intermediate filaments (~10 nm in diameter).²⁴ Although the former two classes have been successfully targeted to develop cancer therapies and/or unique cell biology tools,²⁵ small molecules for IF proteins have not been developed.⁹ Our present results demonstrate that IF proteins can also be targeted with small molecules such as **LBL1**. While **LBL1**'s interaction with LB1 remains to be characterized, its interaction with LA is mediated by the coiled-coil domain. Given the known roles of LA in genome stability and DNA double-strand break (DSB) repair,^{26–27} we found that cells treated with **LBL1** resulted in generation of DSBs as evidenced by phosphorylation of a histone variant H2AX (referred to as γ -H2AX)²⁸ and induction of cellular apoptosis (Figure S7). The unique targeting of **LBL1** shall provide us ample opportunities to further dissect its mechanism of action and also provide us an excellent chemical tool for elucidating lamin biology and generating potential anticancer therapies. Further studies are warranted and will be reported in due course.

MATERIALS AND METHODS

Chemistry. General

Glass Contout solvent purification system was used to purify all the anhydrous solvents to be used for reactions. Melting points were determined in capillary tubes using Mel-Temp and are uncorrected. All ¹H and ¹³C NMR spectra were obtained in a Bruker Avance 400

MHz spectrometer using CDCl₃ or DMSO-*d*₆ as the solvent and the chemical shifts of the residual CHCl₃ (δ 7.24) or DMSO (δ 2.50) were taken as reference. Chemical shifts (δ) are reported in parts per million (ppm), and the signals are described as brs (broad singlet), d (doublet), dd (doublet of doublet), td (triplet of doublet), m (multiplet), q (quartet), s (singlet) and t (triplet). Coupling constants (*J* values) are given in Hz. Silica gel flash chromatography was performed using 230–400 mesh silica gel (EMD). All reactions were monitored using thin-layer chromatography (TLC) on silica gel plates (EMD). Yields were of purified compounds. The mass spectra of the small molecules were obtained from a Thermo Electron LTQ-Orbitrap Discovery high-resolution mass spectrometer (Thermo Scientific) or Advion PlateExpress with electrospray operated either in positive or negative mode. All the final compounds for biological testing were confirmed to be >95% purity based on reverse phase HPLC (Waters, Milford, MA) analysis using an XBridge C18 column (4.6 × 150 mm) and detected at 254 nm. The mobile phases for HPLC are water and acetonitrile, both of which contained 0.1% formic acid.

7-(Prop-2-yn-1-yl)-7*H*-pyrrolo[3,2-*f*]quinazoline-1,3-diamine (**5**)

To a stirred solution of **4**⁷ (400 mg, 2.0 mmol) in dry DMF (24 mL) was added NaH (83 mg, 2.2 mmol). The resulting reaction mixture was stirred at room temperature for 1 h. Then propargyl bromide (169 μL, 2.2 mmol) was added. The reaction mixture was stirred at room temperature for another 2 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel, eluting with 15:1

DCM:MeOH containing 1% DIPEA to give the desired compound **5** (404 mg, 85%) as a pale yellow solid: mp 224–226. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.78 (d, *J* = 8.4 Hz, 1 H), 7.49 (d, *J* = 3.2 Hz, 1 H), 7.09 (d, *J* = 9.2 Hz, 1 H), 7.07 (d, *J* = 3.2 Hz, 1 H), 6.68 (brs, 2 H), 5.70 (s, 2 H), 5.17 (d, *J* = 2.4 Hz, 2 H), 3.44 (t, *J* = 2.4 Hz, 1 H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 162.0, 159.3, 150.3, 129.9, 127.8, 121.0, 119.5, 117.3, 102.6, 101.9, 79.3, 75.8, 35.4; HRESIMS, calcd 238.10872, found 238.10915.

3-Amino-7-(prop-2-yn-1-yl)-7*H*-pyrrolo[3,2-*f*]quinazolin-1-ol (**6**)

A mixture of **5** (194 mg, 0.82 mmol) in 3 N HCl solution (5 mL) was heated under reflux for 3 h. The white solid was collected by filtration and further treated with 1 N NaOH (5 mL) for 30 min at room temperature. The solid was collected by filtration and further purified by column chromatography on silica gel, eluting with 10:1 DCM:MeOH containing 1% DIPEA to give the desired compound **6** (145 mg, 80%) as a white solid: mp >230 °C (dec). ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.84 (d, *J* = 8.8 Hz, 1 H), 7.51 (d, *J* = 3.2 Hz, 1 H), 7.13 (d, *J* = 3.2 Hz, 1 H), 7.10 (d, *J* = 8.8 Hz, 1 H), 6.57 (brs, 2 H), 5.16 (d, *J* = 2.4 Hz, 2 H), 3.44 (t, *J* = 2.4 Hz, 1 H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 163.1, 151.2, 143.5, 131.3, 129.9, 124.8, 117.7, 115.7, 107.9, 102.6, 79.2, 75.9, 35.4; HRESIMS, calcd 239.09274, found 239.09330.

2,5-Dioxopyrrolidin-1-yl-4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzoate (**8**)

NHS (32.5 mg, 0.28 mmol) and EDCI.HCl (54.1 mg, 0.28 mmol) were added to a stirred solution of **7** (50 mg, 0.22 mmol) in dry DMF (1 mL) at 0 °C. The resulting mixture was warmed up to room temperature and stirred for overnight. Then H₂O (10 mL) was added to the reaction mixture. The white solid was collected by filtration to give compound **8** (66 mg,

93%); mp 88–90 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.18–8.15 (m, 2 H), 7.32 (d, *J* = 8.4 Hz, 2 H), 2.92 (s, 4 H); ¹³C NMR (100 MHz, CDCl₃) δ 169.0, 160.9, 135.8, 130.8, 126.7, 126.1, 121.6 (q, *J* = 273 Hz), 28.3 (q, *J* = 41 Hz), 25.6.

***N*-(1-Hydroxy-7-(prop-2-yn-1-yl)-7*H*-pyrrolo[3,2-*f*]quinazolin-3-yl)-4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzamide (9)**

A mixture of **6** (145 mg, 0.61 mmol) and **8** (260 mg, 0.80 mmol) in dry DMF (4 mL) was stirred at 90 °C for 4 h. Then the solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel, eluting with DCM to 20:1 DCM:THF containing 1% DIPEA to give compound **9** as a yellow solid (160 mg, 58%); mp >300 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 13.0–11.9 (m, 2 H), 8.22 (d, *J* = 7.2 Hz, 2 H), 8.03 (d, *J* = 9.2 Hz, 1 H), 7.66 (d, *J* = 2.8 Hz, 1 H), 7.44–7.40 (m, 3 H), 7.25 (d, *J* = 2.4 Hz, 1 H), 5.24 (d, *J* = 2.0, 2 H), 3.48 (s, 1 H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 160.4, 132.4, 131.3, 130.9, 129.4, 126.3, 124.6, 121.8 (q, *J* = 274 Hz), 118.2, 102.8, 79.0, 76.1, 35.5, 28.1 (q, *J* = 40 Hz); HRESIMS, calcd 451.11248, found 451.11291.

***N*-(1-Amino-7-(prop-2-yn-1-yl)-7*H*-pyrrolo[3,2-*f*]quinazolin-3-yl)-4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzamide (LBL1-P)**

To a stirred solution of **9** (135 mg, 0.30 mmol) in dry DMF (5 mL) was added BOP (199 mg, 0.45 mmol) and DBU (90 μl, 0.60 mmol). The resulting reaction mixture was stirred for 7 h at room temperature. Then NH₃ (7 N in MeOH, 1.7 mL, 12 mmol) was added. The reaction mixture was stirred at room temperature for 16 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel, eluting with 8:1 DCM:THF containing 1% TEA to give the desired compound **LBL1-P** (13 mg, 10% yield) as a gray solid: mp >130 °C (dec). ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.7 (s, 1 H), 8.08 (d, *J* = 8.0 Hz, 2 H), 8.04 (d, *J* = 8.4 Hz, 1 H), 7.67 (d, *J* = 2.4 Hz, 1 H), 7.40–7.26 (m, 6 H), 5.28 (d, *J* = 2.0, 2 H), 3.49 (t, *J* = 2.4 Hz, 1 H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 164.5, 162.2, 152.6, 148.5, 136.5, 131.7, 130.6, 128.9, 128.8, 126.4, 121.8 (q, *J* = 273 Hz), 120.6, 120.4, 118.0, 105.5, 102.6, 79.1, 76.1, 35.6, 28.1 (q, *J* = 40 Hz); HRESIMS, calcd 450.12847, found 450.12875.

Plasmids

The LA, LB1 and LB2 expression plasmids were obtained from DNASU repository and subcloned into pEGFP-N3 (Clontech). All the plasmids were sequence-verified by Sanger sequencing. The mammalian expression plasmids encoding different domains of LA were subcloned into pEGFP-N3 (Clontech). The final constructs did not contain the original EGFP sequence. The LA fragments for bacterial expression were subcloned into pET-15b (Novagen). The primers used for subcloning are available upon request.

Cell lines and culture

MDA-MB-231 and MDA-MB-468 were purchased from Developmental Therapeutics Program at the National Cancer Institute. HEK 293T cells were purchased from American Tissue Culture Collection (ATCC). The cells were tested for mycoplasma contamination regularly by PCR. Cells were cultured in high-glucose Dulbecco's Modified Eagle's

Medium (DMEM, Life Technologies) supplemented with 10% FBS (Hyclone) and 10% non-essential amino acids (Life Technologies) at 37 °C with 5% CO₂. All the cells were used within 50 passages.

Cell growth inhibition assay

The cellular growth inhibition assay was carried out as described before.²⁹ The growth inhibition of different cell types was assessed by MTT assay using MTT reagent (Sigma, St. Louis, MO). Briefly, the cells were plated into 96-well plates and the cells were allowed to attach to the bottom of the plates for overnight. Then the cells were treated with different concentrations of different drugs for 72 h. The media were removed and MTT reagent in complete tissue culture media (0.5 mg/mL) was added to each well and incubated at 37 °C for 3 h. The incubation media were removed and 100 µL of DMSO was added to each well. The absorbance of the formed purple formazan solution was read at 570 nm using Packard Fusion plate reader. The percent of growth is defined as $100\% \times (A_{\text{treated}} - A_{\text{initial}}) / (A_{\text{control}} - A_{\text{initial}})$, where A_{treated} represents absorbance in wells treated with a compound, A_{initial} represents the absorbance at time 0 and A_{control} denotes media-treated cells. The GI₅₀ was derived from non-linear regression analysis of the percent of growth-concentration curve in Prism 5.0.

Photocrosslinking and streptavidin enrichment

MDA-MB-468 cells at ~80% confluency were washed once with PBS and then the cells were covered with PBS. The cells were treated with or without **LBL1** for 10 min followed by treatment with **LBL1-P** for 30 min at 37 °C. At the end of incubation, the cells were cooled to 4 °C and UV-irradiated at 365 nm for 5 min. Then the cells were collected by scraping and pelleted by centrifugation at 1000x rpm for 5 min. The cell pellets were washed once with ice-cold PBS. The washed cell pellets were lysed in 1% SDS/PBS with brief sonication followed by centrifugation at room temperature at 14,000x rpm for 10 min. The protein concentration of the supernatant was determined by BCA Protein Assay Kit (Pierce). The crude lysates were adjusted with PBS to give a final concentration of ~1.0 mg/mL for click reaction with biotin-azide using the following condition: azide (50 µM), TCEP (1 mM), TBTA (100 µM), CuSO₄ (1 mM). The mixture was incubated with shaking at room temperature for 1.0 h in the dark. For direct SDS-PAGE analysis, the reaction mixture was separated by SDS-PAGE followed by blotting with HRP-streptavidin (GE-Healthcare, 1:5000). For streptavidin enrichment and protein identification by mass spectrometry, the reaction mixture was further processed as follows. The reaction mixture was mixed with equal volume of MeOH and 0.25 volume of CHCl₃. The mixture was vigorously vortexed for 30 s and centrifuged at 8000x g for 10 min. The supernatant was removed and the protein solid was washed three times with MeOH: CHCl₃ (1:1). Then MeOH was added to the washed solid and briefly sonicated to resuspend the solid. CHCl₃ was added to a final mixture of MeOH:CHCl₃ (4:1). The mixture was vigorously vortexed for 30 s and centrifuged at 14000x rpm for 10 min to pellet the solid. The resulting protein solid was redissolved in PBS with 8 M urea and 2% SDS with brief sonication. The solution was centrifuged at 14000x rpm for 10 min to remove any insoluble material. The proteins in the supernatant was reduced with DTT (10 mM) at 55 °C for 30 min followed by alkylation with iodoacetamide (25 mM) for 30 min at room temperature in the dark. The solution was

diluted 10x with PBS and mixed with PBS-washed streptavidin-agarose beads. The mixture was tumbled at room temperature for 2 h. Then the beads were pelleted by centrifugation at 700x g for 2 min. The beads were extensively washed 3 times in 1% SDS/PBS followed by 6 times in PBS. The bound proteins were then eluted with 1%SDS/PBS containing 1 mM free biotin with heating at 95 °C for 5 min. The supernatant was collected and the beads were further washed with 2 × 200 µL 100 mM NH₄HCO₃. The washes and the supernatant were combined and lyophilized.

Protein LC-MS/MS

Affinity purified proteins from above were dissolved in 20 µL of SDS-PAGE sample buffer, briefly electrophoresed into a SDS-PAGE gel. The top of the gel was excised and all proteins in each sample were subjected to an in-gel digestion with trypsin as previously described³⁰. The tryptic digests were then separated by capillary reverse phase chromatography during a 110 min data-dependent tandem mass spectrometric analysis as previously described³⁰, except using a LTQ Velos Pro linear ion trap mass spectrometer. Data collection used the dynamic exclusion feature of the instrument's control software (repeat count equal to 1, exclusion list size of 100, exclusion duration of 30 sec, and exclusion mass width of -1 to +4) to obtain MS/MS spectra of the 5 most abundant parent ions following each survey scan from m/z 350–2000. The tune file was configured with a 325°C capillary temperature, no averaging of microscans, a maximum inject time of 200 msec and AGC target of 3×10^4 in MS mode and maximum ion time of 100 msec and AGC target of 1×10^4 in MS2 mode. Sequest (Thermo Scientific) was used to search MS2 spectra against a May 2013 version of the Sprot human FASTA protein database, with concatenated sequence-reversed entries to estimate error thresholds. Results were then processed using Python scripts to map peptides to their parent proteins and calculate peptide and protein false discovery as previously described³¹. A minimum of 2 unique peptide sequence matches were required for the identification of proteins and the resulting peptide and protein false discovery rates were approximately 0.4% and 1.5%, respectively.

Transient transfections

HEK293T cells in a well of a 6-well plate were transfected overnight with 1 µg of indicated FLAG-tagged plasmid using Lipofectamine²⁰⁰⁰ (Life Technologies) following the manufacturer's instructions. Then the cells were processed as above for photocrosslinking and clicking with biotin-N₃ and streptavidin pulldown, but with the following exceptions: 1) no reduction or alkylation step was included; 2) the eluted proteins were analyzed by Western blot using anti-FLAG antibody.

Western blot

For western blot analysis, the following primary antibodies were used: anti-LA/C (mouse, Sigma, cat # SAB4200236, 1:2000), anti-FLAG M2 (mouse, Sigma, cat # F4049, 1:4000), anti-Hsp90 (rabbit, Cell Signaling Technology, cat # 4874, 1:1000), anti-LB1 (rabbit, Cell Signaling Technology, cat # 12586, 1:1000), anti-GAPDH (mouse, Santa Cruz Biotechnology, cat # sc-32233, 1:4000), anti-phospho-histone H2AX (rabbit, Cell Signaling Technology, cat # 9718, 1:1200), anti-LB2 (rabbit, Antibody Verify, cat # AAS57463C, 1:200), anti-Histone 3 (H3) (mouse, Cell Signaling Technology, cat#14269, 1:1000). The

HRP-conjugated secondary antibodies were from Cell Signaling Technology or Jackson ImmunoResearch Laboratories.

Protein Purification

GST protein was purified as described previously.³² LA(1–387) and LA(78–387) were expressed and purified from BL21(DE3)pLysS cells. The *E. coli* culture was grown in Luria broth supplemented with ampicillin (50 µg/mL) at 37 °C to an OD₆₀₀ of ~0.6, then IPTG (0.4 mM) was added and the cells were incubated for another 3 h at 37 °C. Then the cells were lysed and the proteins were purified using HisTrap HP columns (GE Healthcare Life Sciences). The binding buffer was buffer A (20 mM TrisHCl, 20% sucrose, pH 7.50) supplemented with 6 M urea. The elution buffer was buffer A supplemented with 6 M urea and 10–300 mM imidazole. The purified proteins were snap frozen in liquid nitrogen and stored at –80 °C. To prepare the proteins for binding experiments, the proteins were dialyzed into HBS (20 mM HEPES, 250 mM NaCl, pH 8.0) at 4 °C.

Limited proteolysis

The purified proteins (0.1 mg/mL) in HBS were incubated with different concentrations of **LBL1** in a PCR reaction plate at 20 °C for 30 min, when sequencing-grade trypsin (Sigma) was added to give a ratio of trypsin/protein = 1/500. Proteolysis was allowed for 20 min at 20 °C. The reaction was quenched by adding SDS-PAGE buffer and the samples were analyzed by SDS-PAGE followed by Coomassie blue staining.

Thermal shift assay (TSA)

Different concentrations of **LBL1** were incubated with LA(1–387) (0.3 mg/mL) or GST (2 µM) for 30–60 min at room temperature. Then 5x Sypro Orange (Life Technologies) was added. Twenty µL/well was aliquoted into a qPCR plate (Life Technologies). Then the TSA assay was performed in QuantStudio 7 Flex (Life Technologies) at a ramping rate of 0.05 °C/s. The T_m was calculated from the peak of the first derivative of the fluorescence intensity-temperature plot.

Subcellular fractionation

For subcellular localization of the crosslinked proteins, the UV-irradiated cells were lysed in a hypotonic buffer (25 mM TrisHCl, 5 mM KCl, 0.5 mM DTT, pH 7.40) supplemented with protease inhibitor cocktail (Pierce) and 1 mM PMSF for 10 min on ice. Then the cells were homogenized with a dounce homogenizer (15 strokes). The supernatant was separated by centrifuging at 600x g for 10 min at 4 °C. The supernatant was further centrifuged at 14000x rpm for 20 min at 4 °C and the supernatant was collected as cytosolic fraction. The nuclear pellets obtained from initial 600x g centrifugation were washed 5 times with an isotonic buffer (10 mM TrisHCl, 6 mM MgCl₂, 0.5% Triton X-100, 10% glycerol, pH 7.40). The washed nuclear pellets were then lysed in 1%SDS/PBS with brief sonication followed by centrifugation at 14000x rpm for 10 min at room temperature. The supernatant was collected as nuclear fractions. The protein concentrations of both cytosolic and nuclear fractions were determined by BCA Protein Assay Kit. Equal amount of proteins were then clicked with rhodamine-N₃ using the same click reaction condition as above. The reaction mixture was

then separated on a SDS-PAGE for in-gel fluorescence gel scanning with ChemiDoc-MP (Bio-Rad).

In-cell click reaction

MDA-MB-231 cells growing on the cover slips were washed once with PBS and then the cells were covered with PBS. The cells were treated with DMSO or **LBL1** for 20 min followed by **LBL1-P** for 20 min. After UV-irradiated for 5 min, the cells were fixed with 3.7% formaldehyde and permeabilized with 0.5% triton X-100. After the cells were washed with 3% BSA in PBS, the cells were incubated with Click-iT[®] Cell reaction cocktail (Life Technologies) supplemented with rhodamine-N₃ for 30 min at room temperature. Then the cells were washed with 3% BSA in PBS before being incubated with anti-LA (mouse, Sigma, cat# SAB4200236, 1:1000) overnight. The cells were then incubated with Alexa Fluor 488 donkey anti-mouse secondary antibody for one hour at room temperature and the coverslips were mounted as above. The coverslips were then mounted in ProLong Gold AntiFade Reagent with DAPI (Life Technologies) and the images were acquired with a fluorescence microscope ApoTome (Zeiss). For colocalization analysis, the Z-stack of images were reconstructed and colocalization Pearson correlation coefficients were determined using the Coloc module in the Imaris software package (Bitplane).

Apoptosis analysis

MDA-MB-231 cells were treated with indicated concentrations of **LBL1** for 5 days. Then the cells were collected by trypsinization and washed twice with ice-cold PBS. The cells were resuspended in 1x binding buffer (BD Pharmingen, Annexin V-FITC Apoptosis Detection Kit I) and stained with Annexin V-FITC and PI at room temperature according to the manufacturer's protocol. The stained cells were analyzed by BD FACSCANTO II.

Supporting Information available

Synthesis of compounds **1–3**, Table S1 and Figures S1–S6 are available in the online version.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Overview of a target identification approach using **LBL1-P**. (A) Chemical structures of **LBL1** and **LBL1-P**. (B) Schematic diagram of the chemoproteomics approach using a clickable photocrosslinking probe **LBL1-P**.

Figure 2.

Identification of lamins as the targets of **LBL1**. (A) **LBL1-P** labeling in MDA-MB-468 cells. The cells were treated with **LBL1-P** with or without **LBL1**. Then the cells were UV-irradiated followed by cell lysis and click reaction with a biotin- N_3 . The lysates were then separated on a SDS-PAGE gel for streptavidin blotting. (B) Mass spectrometry identification of lamins as the targets of **LBL1**. The spectra counts of identified proteins in the presence and in the absence of **LBL1** were plotted on the x- and y-axis, respectively. (C) **LBL1-P**-labeled proteins were localized in the nucleus. The cells were treated as in (A) except that the cells were fractionated into cytosolic and nuclear fractions. Then the individual fractions were clicked with a rhodamine- N_3 for in-gel fluorescence imaging. The same gel was also used for western blotting with indicated antibodies. C, cytosol; N, nucleus.

Figure 3.

Validation of lamins as the targets of **LBL1**. **(A)** **LBL1** bound to nuclear lamins. The photocrosslinked and biotin-N₃ clicked cell lysates were pulled down with streptavidin beads. The precipitated proteins were separated on SDS-PAGE and probed with indicated antibodies. **(B)** **LBL1-P** bound to LA and LB1 with higher efficiency than LB2. HEK293T cells were transfected with indicated FLAG-tagged plasmid. Then the cells were treated as in **(A)** except that anti-FLAG was used. SE, short exposure; LE, long exposure. IB: immunoblot. **(C)** **LBL1** bound to LA in living cells. The cells were treated with **LBL1-P** with or without **LBL1** followed by UV irradiation and in-cell click reaction with a rhodamine-N₃. The cells were then analyzed by fluorescence microscopy after anti-LA staining. **(D)** Shown are the deconvoluted Z-stacks of images of the cells treated with **LBL1-P** alone as shown in **(C)**. “Colocalization” was presented as a new channel to specifically label the colocalization between **LBL1-P** and LA.

Figure 4.

LBL1 binds to LA(1-387). **(A)** The domain organization of LA and different truncation constructs prepared. The **LBL1-P** labeling efficiency of these constructs is indicated on the right. **(B)** Representative blots to show that **LBL1-P** labeled LA(1-387). HEK293T cells were transfected with indicated FLAG-tagged LA fragments. Then the cells were treated as in Figure 3B. **(C–D)** The indicated proteins were incubated with different concentrations of **LBL1**. Then trypsin was added to initiate partial proteolysis. The residual proteins were analyzed by SDS-PAGE followed by coomassie blue staining. **(E–F)** **LBL1** binds to LA(1-387) as assessed by TSA. LA(1-387) was incubated with different concentrations of **LBL1**. Then Sypro Orange was added and the fluorescence intensity of the samples was measured during gradual heating.

Scheme 1.
Synthesis of **LBL1-P**.

Table 1Growth inhibitory activities of **LBL1** and its analogs in breast cancer cell lines.^a

			GI ₅₀ (μM)

compd	R ₁	R ₂	MDA-MB-468	MDA-MB-231
LBL1		-H	0.44 ± 0.14	1.60 ± 0.51
1			1.19 ± 0.18	3.27 ± 0.38
2		-H	6.99 ± 2.98	14.13 ± 4.99
3		-H	0.97 ± 0.11	9.24 ± 1.90

			GI ₅₀ (μM)

compd	R ₁	R ₂	MDA-MB-468	MDA-MB-231
LBL1-P			3.71 ± 0.07	6.71 ± 1.21

^aThe growth inhibitory activities of the compounds are presented as the mean GI₅₀ ± SD of at least two independent experiments in duplicates.

The GI₅₀ is the concentration needed to inhibit the cell growth by 50% after incubating the cells with the compounds for 72 h.