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Chemical affinity matrix-based identification of prohibitin as a binding protein to anti-resorptive sulfonyl amidine compounds

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ABSTRACT

In order to identify the binding proteins to anti-resorptive 5-chloro-1-(2,6-dimethylpiperidin-1-yl)-*N*-tosylpentan-1-imine (**1**), the chemical affinity matrix for the compound **1** (**2b**) was designed and synthesized. Using **2b**-based chemical proteomics, prohibitin was identified as one of strong binding proteins for **2b**.

The identification of target or binding proteins for bioactive small molecules including new therapeutics can extend our understanding of their action mechanism (or responses) and provide the biochemical evidence for the prediction of their side effects in the body. Furthermore, the discovery of new drugable target protein(s) through the process of target identification may offer an improved therapeutic benefit by accelerating the development of new therapeutics based on them. Therefore, the chemical proteomics with a fundamental goal to identify target (or binding) proteins for bioactive small molecules is an important field in the view of drug discovery and development as well as the basic research although it is required for the advanced, complicated and integrated tools and technologies for the purification, enrichment, identification and validation of target proteins.[1,2]

Osteoclastogenesis is the overall process of hematopoietic stem cells to differentiate into mature osteoclasts that resorb the bone matrix, but the resorptive area is filled with bone matrix synthesized by osteoblasts.[3,4] This is referred as bone remodeling. However, an imbalance in bone remodeling caused by increased bone resorption over bone formation due to several factors such as menopause leads to the decrease of bone mineral density and it subsequently increases the risk of factures that is a major cause of mortality in the patients with bone disorders such as osteoporosis.

Previously, we reported that sulfonyl amidine derivatives exhibited anti-resorptive activities to inhibit the osteoclastogenesis.[5] Among the sulfonyl amidine derivatives, 5-chloro-1-(2,6-dimethylpiperidin-1-yl)-*N*-tosylpentan-1-imine (**1**) was proved as a potent anti-resorptive agent with the IC_{50} value of 1.75 ± 0.26 μM (Fig. 1).

To identify the target protein of the amidine compound **1**, a chemical affinity matrix **2b** was designed (Fig. 1). Several approaches have been developed to purify/enrich and identify target (or binding) proteins/peptides.[1] Among them, the photoaffinity probe can strongly and irreversibly bind to its target proteins,

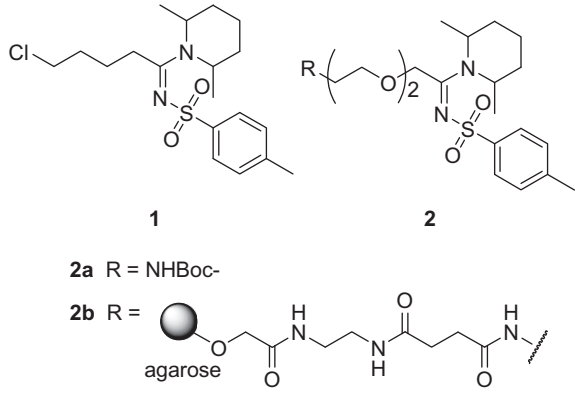

Figure 1. Structure of chemicals.

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but it could also crosslink with the non-specific proteins. Additionally, the introduction of a photoreactive moiety and a tag such as biotin into the structure of bioactive small molecule causes the decrease of biological activity and any problem due to the condition to separate the protein–probe covalent complex in the affinity column (e.g. normally, breaking the avidin–biotin interaction requires 6–8 M guanidine–HCl, pH 1.5) during analysis by MALDI should be avoided by dilution or washing of the protein sample. Therefore, in this study, the chemical affinity matrix-based approach was simply introduced as the tool to fish out the binding partners. Generally, the biological activity of small molecule can be decreased by introducing a linker for target protein identification. So the appropriate decision of linker's size and the site for its attachment are critical for the design of the chemical affinity matrix. When the 2,6-dimethylpiperidine moiety of amidine **1** was replaced with diisopropylamino group, the resulting amidine compound showed no anti-resorptive activity (105% TRAP activity of control at 60 μM) implying that the 2,6-dimethylpiperidine moiety is necessary for its anti-resorptive activity. TRAP activity was evaluated as described previously.[5] Therefore, the 2,6-dimethylpiperidine moiety was retained for the chemical affinity matrix. Next, the 4-chlorobutyl moiety was changed into *tert*-butyl 2-(2-methoxyethoxy)ethylcarbamate group, which can generate the amine group for the coupling with the Affi-Gel, and the resulting amidine **2a** showed comparable inhibitory effect on TRAP activity with the IC_{50} value of 6.89 ± 0.69 μM to the compound **1** (Fig. 1).

Therefore, the chemical affinity matrix (**2b**; Fig. 1) containing 2-(2-methoxyethoxy)ethyl linker coupled to Affi-Gel 10 was designed and synthesized as described previously with modification (Scheme 1).[6–8] Then, in order to identify the proteins that bind to the chemical affinity matrix, **2b** was incubated with RAW264.7 cell lysate and then the bound proteins were resolved by SDS–PAGE as shown in Figure 2A. Arrow-indicated proteins (T1–T5) were identified by LCQ mass spectrometry peptide mass fingerprinting after in-gel digestion as described previously (Table 1).[9] Since among 5 candidate proteins binding to **2b**, prohibitin had higher Mascot score (446) than others, we further evaluated whether prohibitin could interact with **2b** by western blot analysis.[10] As shown in Figure 2B, prohibitin was strongly detected in the bound fraction

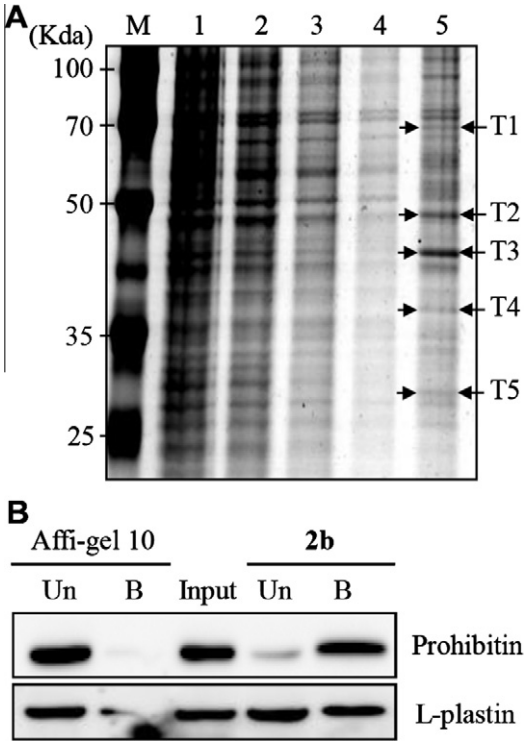
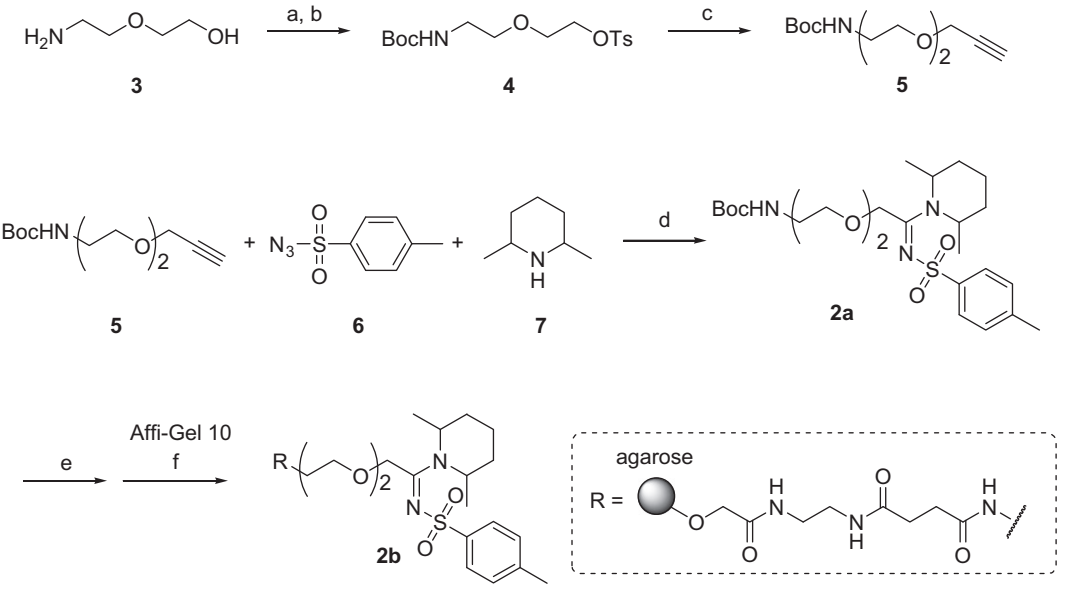


Figure 2. Identification of putative **2b**-binding proteins in RAW264.7 cell lysate. Putative **2b**-binding proteins was identified in Commassie Blue-stained gel compared to unbound (lane 1), subsequent washing fractions (lanes 2–4) and boiled (or bound) fraction (lane 5). The left lane (M) was the standards of molecular weight. (B) Prohibitin levels in unbound (Un) and bound (B) fractions of non-conjugated Affi-Gel 10 beads and **2b** were evaluated by western blot analysis. Same amount of cell lysate were incubated with non-conjugated Affi-Gel 10 beads or **2b**.



Scheme 1. Synthesis of chemical affinity matrix **2b**. The amino group of 2-(2-aminoethoxy)ethanol (**3**) was protected with Boc group, and then hydroxyl group was tosylated. The resulting tosylated compound **4** was reacted with propargyl alcohol in the presence of NaH to give the linker part **5** containing terminal alkyne moiety. The terminal alkyne compound **5**, sulfonyl azide compound **6**, and amine compound **7** gave sufonyl amidine compound **2a** in the presence of Cu catalysis at room temperature in 57% yield.[6] After deprotecting of Boc group with trifluoroacetic acid, the resulting amino group was coupled with Affi-Gel in the presence of diisopropylethylamine to give the final chemical affinity matrix **2b**.[7] Reagents and conditions: (a) $(Boc)_2O$ (1.0 equiv), Et_3N (1.0 equiv), Dioxane, 0 °C to rt, 12 h, 99%; (b) TsCl (1.5 equiv), Et_3N (3.0 equiv), CH_2Cl_2, 0 °C to rt, 4 h, 85%; (c) 60% NaH (10 equiv), propargyl alcohol (10 equiv), THF, 0 °C to rt, 2 d, 82%; (d) CuI (0.2 equiv), THF, rt, 3 h, 57%. (e) TFA (15 equiv), CH_2Cl_2, rt 1 h, 99%; (f) conjugation with Affi-Gel 10.[7]

Table 1
Candidate proteins binding to **2b**

No.	Accession number	Protein name	MASCOT score
T1	GI31543113	Lymphocyte cytosolic protein 1 (L-plastin)	269
T2	GI55491	Unnamed protein product	381
T3	GI809561	Gamma-actin	410
T4	GI56188	Glyceraldehyde 3-phosphate-dehydrogenase	292
T5	GI4505773	Prohibitin	446

of **2b** suggesting that prohibitin could be one of specific binding proteins to **2b**. Although L-plastin with low Mascot score was detected in both the unbound and bound fraction of **2b**, we could not conclude that L-plastin is the unspecific binding protein to **2b** because it still exhibited the binding potential to **2b**. Binding kinetics study based on surface plasmon resonance (SPR) would access their binding potentials to **2b** in a further study.

Prohibitin is ubiquitous, evolutionarily conserved protein that is mainly localized and functions in mitochondria.[11] The functional role of prohibitin in development, senescence and tumor suppression has been also proposed, but there was no evidence to elucidate the functional involvement of prohibitin in the osteoclastogenesis. However, its repressive role in the transcriptional activity of estrogen receptor α (ERα) made us suggest the hypothesis that sulfonyl amidine derivatives with anti-resorptive activities might lead to the activation of the estrogen signaling by the inhibition of prohibitin to bind with estrogen receptor that is consequently activated and suppress the osteoclastogenesis.[12] Indeed, the osteoclastic ERα-mediated osteoprotective action of estrogens has been reported in several studies. The functional involvement of prohibitin in osteoclastogenesis and its relationship with estrogen signaling as well as its binding kinetics with **2b** would be accessed in a further study.

The expression of L-plastin (also referred to L-fimbrin) that is localized in membrane ruffles and cell adhesion sites has been shown to be restricted to cells of the haematopoietic lineage including macrophages, but the sequential expression and differential localization of fimbrin isoforms during epithelial cell differentiation has been also reported.[13] Interestingly, L-plastin, an actin-bundling protein, has been shown to be a component of the osteoclast adhesion complexes called podosomes that is rare in the primarily mononucleated cells, but commonly found at the periphery of multinucleated cells indicating that the number of cells containing L-plastin-positive podosomes increased during osteoclastogenesis.[14] Therefore, the inhibition of L-plastin might affect the formation and functional role of podosomes in the early stage of osteoclastogenesis; in turn, it could block the formation of multinucleated osteoclasts. After confirming the binding potential of **2b** to L-plastin, its functional involvement in the osteoclastogenesis might be evaluated.

In conclusion, we here designed and synthesized **2b** as the chemical affinity probe for the sulfonyl amidine derivatives with anti-resorptive activity, and by using **2b**-based chemical proteomics, prohibitin was identified as one of binding proteins for **2b**. Further studies such as SPR-based binding kinetics, gene silencing and X-ray co-crystallography could be required for validating the interaction between bioactive small molecule and its binding proteins, and elucidating the functional involvement of the binding proteins in the biological event of interest.

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7. Conjugation of **2a** with Affi-Gel 10 was performed as the following; Affi-Gel 10 (0.5 ml; Bio-Rad) was transferred into a 3-ml cartridge with 20mPE FRIT (Applied Separations). The supernatant solvent was drained and Affi-Gel 10 was washed with DMSO three times. After capping the cartridge with a stopper, a solution of 3.75 μmol of **2a** in 0.5 ml DMSO and 50 μl of *N*,*N*-diisopropylethylamine (DIEA) were added to the gel. After shaking for 3 h at room temperature, the slurry was drained and the gel was washed with DMSO three times. Then, 1 ml DMSO with 50 mM ethanolamine and 15 μl of DIEA were added to the gel. After shaking for 3 h at room temperature, the slurry was drained and the gel was washed with DMSO three times and saturated with buffer A (25 mM Tris–HCl, pH 7.4, 0.25 M sucrose, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM PMSF, 1% Nonidet P-40 and protease inhibitor cocktail).
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9. Kim, M. H.; Choi, Y. L.; Heo, J.-N.; Min, Y. K.; Kim, S. H. *Bull. Korean Chem. Soc.* **2010**, *31*, 2047; Protein sample preparation and its incubation with **2b**. RAW264.7 cells were homogenized (1:5, w/v) in buffer A under sonication. The homogenate was then centrifuged at 10,000*g* for 15 min and 0.5 ml of the supernatant diluted with buffer A (7 mg/ml) was stirred with buffer A-saturated **2b** at 4 °C for 15 h. After incubation, the resins were precipitated by centrifugation at 10,000*g* for 1 min and washed three times with 1 ml of buffer A. The washed beads were then resuspended with 20 μl of SDS sample buffer (100 mM Tris–HCl, 2% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 2% glycerol, 0.01% bromophenol blue, pH 7.6), incubated at 25 °C for 10 min, and subjected to SDS–PAGE followed by the gel staining.
10. Western blot analysis. RAW264.7 cell lysate (40 μg) were incubated with non-conjugated Affi-Gel 10 or **2b** at 4 °C for 15 h and centrifuged at 10,000*g* for 1 min. The supernatant (unbound fractions) was denatured with SDS sample buffer. Then, the pellets were washed twice with buffer A, resuspended with 20 μl of SDS sample buffer and incubated at 25 °C for 10 min for getting bound fractions. Denaturation of unbound and bound fractions was done by boiling and then samples were subjected to 10% polyacrylamide gels. Electrophoresis was performed using the Mini Protean 3 Cell (Bio-Rad). The resolved proteins were transferred to PVDF membrane (Millipore). The membranes were incubated in blocking buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 3% nonfat dry milk) and then incubated with diluted primary antibodies (1:1000) for 2 h at room temperature. Antibodies used in this study were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz). Following the primary antibody reactions, the membranes were washed with blocking buffer three times (15 min each) and then probed with diluted secondary antibodies (1:2000) for 1 h. The membranes were washed three times (15 min each) and developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) using the LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd).
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