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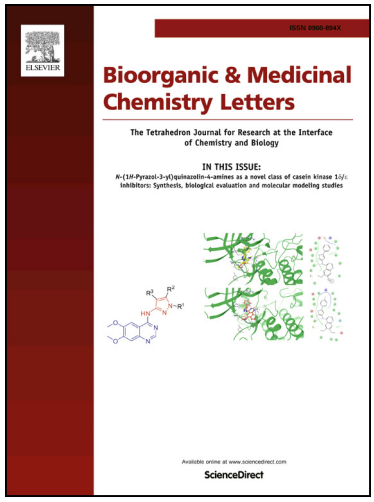

Simple and efficient knockdown of His-tagged proteins by ternary molecules consisting of a His-tag ligand, a ubiquitin ligase ligand, and a cell-penetrating peptide

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Simple and efficient knockdown of His-tagged proteins by ternary molecules consisting of a His-tag ligand, a ubiquitin ligase ligand, and a cell-penetrating peptide

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KEYWORDS: protein knockdown; His-tag-fused protein; ubiquitin-proteasome system; carrier peptide

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Abstract

We designed and synthesized hybrid molecules for a protein knockdown method based on the recognition of a His-tag fused to a protein of interest (POI). The synthesized target protein degradation inducers contained three functional moieties: a His-tag ligand (nickel nitrilotriacetic acid [**Ni-NTA**]), an E3 ligand (bestatin [**BS**] or **MV1**), and a carrier peptide (**Tat** or nonaarginine [**R9**]). The designed hybrid molecules, **BS-Tat-Ni-NTA**, **MV1-Tat-Ni-NTA**, **BS-R9-Ni-NTA**, and **MV1-R9-Ni-NTA**, efficiently degraded His-tagged cellular retinoic acid binding protein 2 via the ubiquitin-proteasome system (UPS). This system will become a useful tool for research into selective protein degradation inducers that act via the UPS.

Elucidating the functions of intracellular proteins is quite important for the development of innovative drugs, and the artificial control of protein expression is a useful technique for clarifying protein functions. A protein knockdown strategy in which the degradation of proteins of interest (POI) is induced via the ubiquitin-proteasome system (UPS) has recently attracted attention. Based on this approach, novel small molecules composed of two different ligands connected by a linker (one is a ligand for the POI, and the other is a ligand for ubiquitin ligases [E3]) have been developed. These molecules, which are called proteolysis-targeting chimeras (PROTAC)[1-9] or specific and non-genetic inhibitors of apoptosis protein [IAP]-dependent protein erasers (SNIPER)[10-21] are able to induce the ubiquitylation and subsequent proteasomal degradation of POI via the UPS, and a variety of PROTAC and SNIPER that target pathogenic proteins have been reported.

We have recently described the development of a small hybrid molecule, **SNIPER(CH6)**, which mediates the degradation of CH6 tag-fused proteins.[22-26] The CH6 tag is a modified version of the conventional His-tag and consists of hexa-His and a Cys residue. It attaches to the N- or C-terminus of the POI. **SNIPER(CH6)** is composed of three functional moieties, nickel nitrilotriacetic acid (**Ni-NTA**; a His-tag ligand), a maleimide moiety covalently conjugated to the Cys residue in the CH6 tag, and the IAP antagonist (**MV1**) as an E3 ligand. However, **SNIPER(CH6)** itself cannot penetrate into cells, and therefore, **CPP-His**, a cell-penetrating peptide (CPP) conjugated with a His-tag was used to deliver **SNIPER(CH6)** into cells. As expected, the combination of **SNIPER(CH6)** and **CPP-His** efficiently degraded CH6-tagged POI within cells.[26] This system could be an effective screening tool for finding proteins that are susceptible to degradation via the UPS and for identifying target proteins for drug discovery. However, there are two main disadvantages of this method. The first disadvantage is that it

involves the use of two types of molecules; i.e., **SNIPER(CH6)** and **CPP-His**, and the other concerns the fact that **SNIPER(CH6)** is also decomposed by the UPS together with the CH6-tagged POI because **SNIPER(CH6)** covalently binds to the protein (Figure 1).

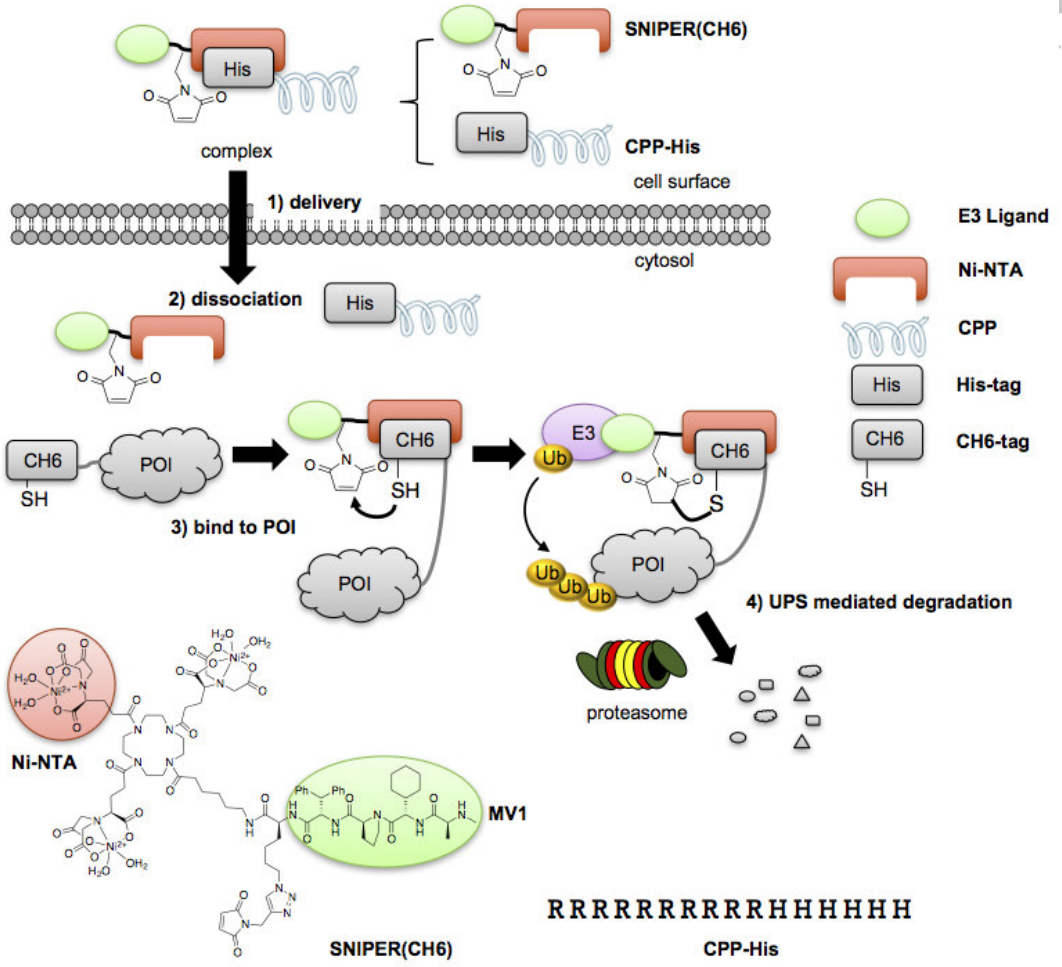


Figure 1. Mechanism responsible for the degradation of CH6 tag-fused POI using a combination of **SNIPER(CH6)** and **CPP-His**[24]

The **SNIPER(CH6)/CPP-His** complex penetrates the plasma membrane efficiently. In the cytosol, **SNIPER(CH6)** is released from **CPP-His** and forms another complex with the CH6-tagged POI through the covalent binding of its maleimide moiety with the Cys residue of the CH6 tag. Finally, the POI is ubiquitylated and degraded via the UPS.

In this study, we designed a new type of His-tagged POI degradation inducer. The synthesized hybrid molecules were composed of three functional moieties, a His-tag ligand (**Ni-NTA**), an IAP antagonist (bestatin [**BS**][10,12] or **MV1**[19,26]) as an E3 ligand, and a representative carrier peptide (**Tat** or nonaarginine [**R9**]). The mechanism proposed for the degradation of His-tagged POI is shown in Figure 2. 1) The degradation inducer **MV1-CPP-Ni-NTA** is taken up into the target cells. 2) In the cells, the degradation inducer non-covalently binds to the His-tag moiety of the POI. 3) Finally, E3 is recruited to the E3 ligand, and the POI is ubiquitylated and degraded by the UPS. The designed hybrid molecules, **BS-Tat-Ni-NTA**, **MV1-Tat-Ni-NTA**, **BS-R9-Ni-NTA**, **MV1-R9-Ni-NTA**, were synthesized, and their His-tagged POI degradation activities were evaluated.

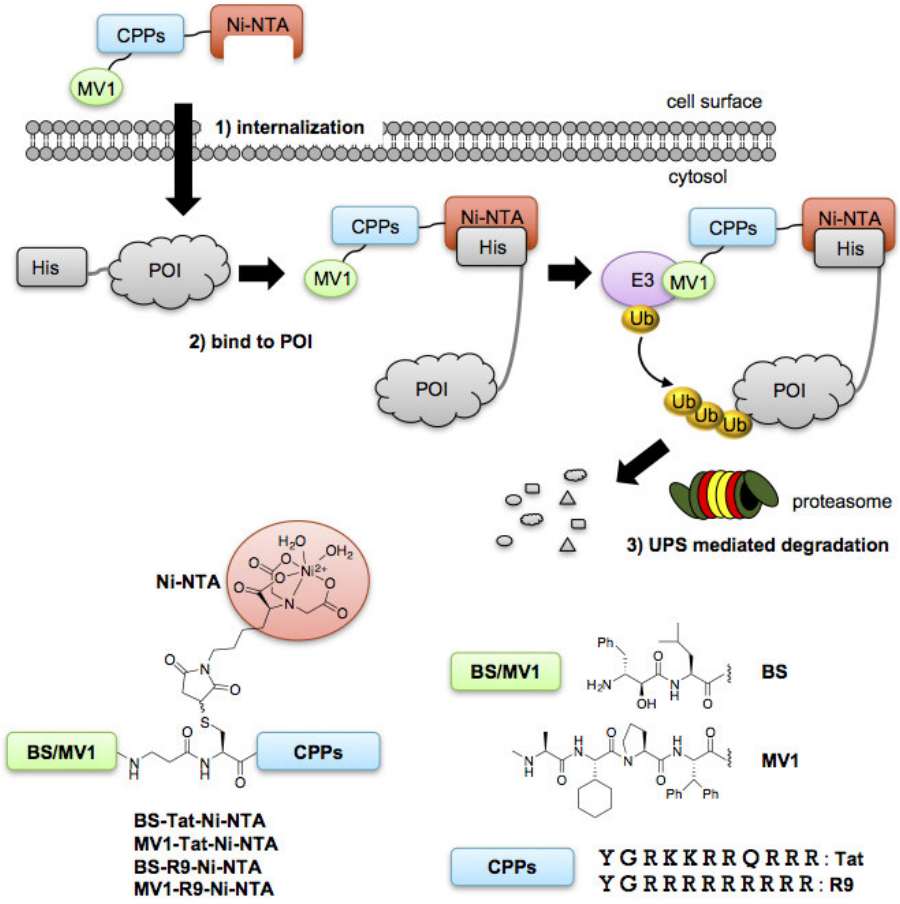
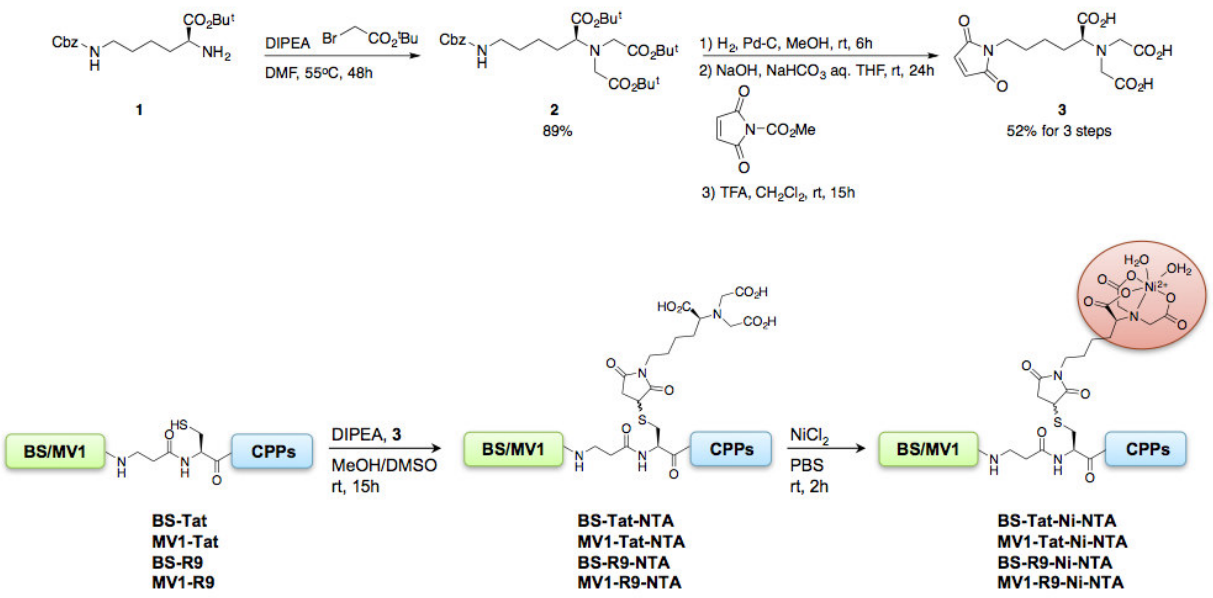


Figure 2. Proposed mechanism for the degradation of His-tag-fused POI using a hybrid molecule (see the main text for details)

The synthetic route for the designed molecules is shown in Scheme 1. The **Ni-NTA** moiety **3** was synthesized starting from the lysine derivative **1**. Carrier peptides containing **BS** or **MV1**, **BS-Tat**, **MV1-Tat**, **BS-R9**, **MV1-R9** were prepared via the conventional solid-phase peptide synthesis method. The conjugation of the Ni-NTA moiety **3** with the abovementioned peptides was performed under basic conditions, producing **BS-Tat-NTA**, **MV1-Tat-NTA**, **BS-R9-NTA**, and **MV1-R9-NTA**, and then a nickel ion (Ni^{2+}) was added to each molecule to give the desired

molecules; i.e., **BS-Tat-Ni-NTA**, **MV1-Tat-Ni-NTA**, **BS-R9-Ni-NTA**, and **MV1-R9-Ni-NTA**.[27]



Scheme 1. Synthesis of the His-tagged POI degradation inducers **BS-Tat-Ni-NTA**, **MV1-Tat-Ni-NTA**, **BS-R9-Ni-NTA**, and **MV1-R9-Ni-NTA**

Then, we evaluated the effects of the synthesized compounds on 10×His-cellular retinoic acid binding protein 2 (CRABP2)-T7 protein degradation in HT1080 cells by comparing with the protein level of mock treatment (lane 1) (Figure 3).[28,29] As demonstrated in previous studies, treatment with the IAP antagonist (**BS**[10,12] or **MV1**[19,24]) moiety-containing molecules resulted in a reduction in the level of the c-IAP1 protein. The control compound, **MV1-all-trans retinoic acid (ATRA)**,[19] which targets the CRABP2 protein, induced the degradation of the 10×His-CRABP2-T7 protein (compare lane 3 with lane 1), which was blocked by the addition of the

proteasome inhibitor MG132 (compare lane 4 with lane 3). Treatment with the carrier peptide **Tat** alone did not affect the expression levels of c-IAP1 or the 10×His-CRABP2-T7 protein (compare lane 21 with lane 1). A slight reduction in the protein level was observed in the cells treated with 10 μM **BS-Tat-Ni-NTA** (compare lane 5 with lane 1), whereas treatment with 3 μM **MV1-Tat-Ni-NTA** successfully induced protein degradation (compare lane 9 with lane 1). A similar tendency was observed for 10 μM **BS-R9-Ni-NTA** (compare lane 13 with lane 1) and 3 μM **MV1-R9-Ni-NTA** (compare lane 17 with lane 1). Thus, the MV1-conjugated molecules (**MV1-Tat-Ni-NTA**, **MV1-R9-Ni-NTA**) showed higher activities than BS-conjugated ones (**BS-Tat-Ni-NTA**, **BS-R9-Ni-NTA**) in lower concentration (3-5 μM for MV1-conjugated molecules and 10 μM for BS-conjugated molecules, respectively). These results were consistent with those of a previous study, in which it was demonstrated that the E3 ligand activity of **MV1** was greater than that of **BS**.[19] Furthermore, **BS-R9-Ni-NTA** and **MV1-R9-Ni-NTA**, which contained **R9** (lanes 13 and 17), exhibited greater activity than **BS-Tat-Ni-NTA** and **MV1-Tat-Ni-NTA**, which contained the Tat peptide (lanes 5 and 9), indicating that **R9** is a superior carrier for transporting cargo molecules into HT1080 cells. The reductions in the levels of CRABP2 and c-IAP1 induced by the synthesized compounds were suppressed by MG132 (compare the protein levels in the presence of MG132 with in the absence of MG132), indicating that these compounds also induced proteasomal degradation of the target proteins.

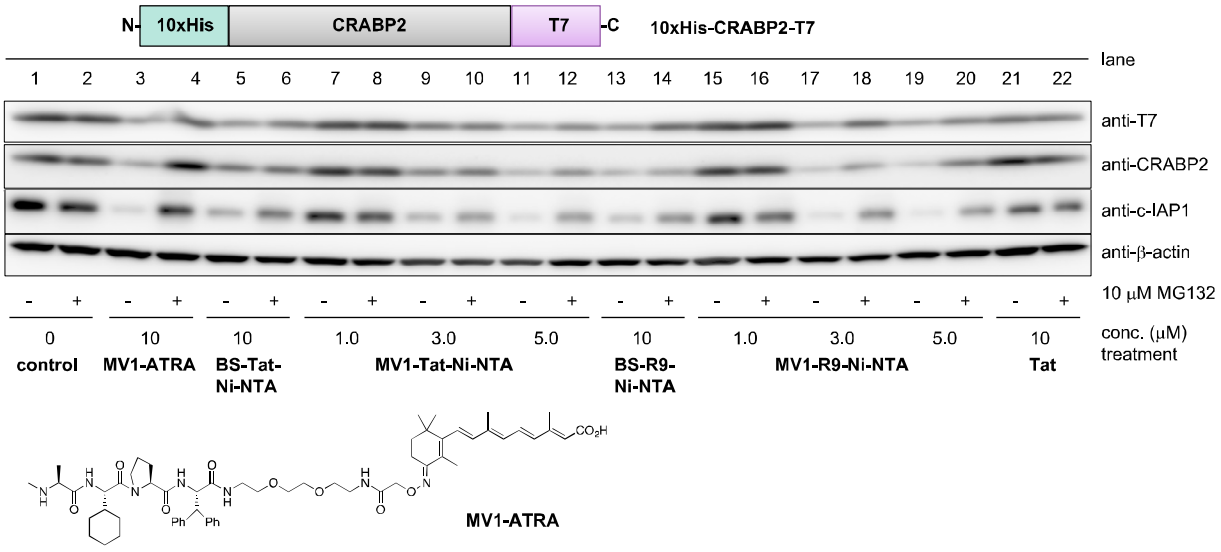


Figure 3. The degradation of 10×His-CRABP2-T7 by the synthesized compounds

HT1080 cells that constitutively expressed 10×His-CRABP2-T7 were treated with the indicated dose of the relevant compound with or without MG132 for 6 h. The lysates were analyzed with the indicated antibodies.

In summary, we developed a protein knockdown method for His-tagged POI involving the use of a hybrid degradation inducer containing three components, a His-tag ligand (**Ni-NTA**), an E3 ligand (**BS** or **MV1**), and a carrier peptide (**Tat** or **R9**). The compound **MV1-R9-Ni-NTA** efficiently induced the degradation of His-tagged CRABP2 proteins. This method will become a useful technique for research into selective UPS-based protein degradation inducers. The development of innovative protein degradation inducers that target various types of proteins is currently underway.

Acknowledgements

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27. See the supplementary materials.
28. HT1080 cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, and 100 μg/ml of kanamycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cells were transfected with the expression vectors for 10×His-CRABP2-T7 using Lipofectamine 2000 (Invitrogen), according to the

manufacturer's instructions. After 48 h, the cells were selected with 500 μg/ml G418 (Roche) for 2 weeks to isolate single-cell clones.

29. The cells were treated as described in the figure legends, washed with phosphate-buffered saline (-), lysed in sodium dodecyl sulfate (SDS) lysis buffer (1% SDS, 0.1 M Tris-HCl, and 10% glycerol; pH 7.4), and boiled for 10 min. Protein concentrations were determined using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific) and normalized to the total protein concentration of each lysate. After being boiled for 5 min with Laemmli buffer, each lysate was resolved via SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 5-20% gradient gel, before being transferred onto a polyvinylidene difluoride membrane. After being blocked with Tris-buffered saline-Tween 20 (25 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20; pH 7.4) containing 5% skimmed milk, the membranes were probed with the antibodies as described in the figure legends. The immunoblots were visualized via enhanced chemiluminescence with the Clarity western ECL substrate (BIO-RAD).

Graphical Abstract

Simple and efficient knockdown of His-tagged proteins by ternary molecules consisting of a His-tag ligand, a ubiquitin ligase ligand, and a cell-penetrating peptide

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