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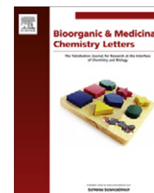

Double protein knockdown of cIAP1 and CRABP-II using a hybrid molecule consisting of ATRA and IAPs antagonist

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Protein knockdown can be achieved by the use of a small molecule that possesses affinity for both the target protein and ubiquitin ligase. We have designed such a degradation-inducing molecule targeting cIAP1 and CRABP-II, which are involved in proliferation of several cancer cell lines and in neuroblastoma growth, respectively. As a CRABP-II-recognizing moiety, *all*-trans retinoic acid (ATRA, **3**), a physiological ligand of CRABP, was chosen. As a cIAP1-recognizing moiety, MV1 (**5**), which is a cIAP1/cIAP2/XIAP pan-ligand, was chosen. Although cIAP1 itself possesses ubiquitin ligase activity, we expected that its decomposition would be efficiently mediated by related molecules, including cIAP2 and XIAP, which also possess ubiquitin ligase activity. The designed degradation inducer **6**, in which ATRA (**3**) and MV1 (**5**) moieties are connected via a linker, was synthesized and confirmed to induce efficient degradation of both cIAP1 and CRABP-II. It showed potently inhibited the proliferation of IMR32 cells.

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Inhibitor of apoptosis proteins (IAPs) are overexpressed in certain tumor cells and inhibit apoptosis induced by a variety of stimuli.[1–4] X-linked inhibitor of apoptosis protein (XIAP) and cellular inhibitor of apoptosis protein 1 and 2 (cIAP1 and cIAP2) show ubiquitin ligase (E3) activity and they promote proteasomal degradation of their substrate proteins, including caspase, which is an effector of apoptosis.[5,6] Therefore, inhibition of these IAPs is a potential strategy for cancer treatment.[7,8] The mitochondrial protein Smac (second mitochondria-derived activator of caspases) is an endogenous IAPs antagonist that binds to the BIR domain of IAP proteins. Potent and cell-permeable small-molecular IAP antagonists, mimicking Smac's AVPI tetrapeptide sequence, have provided useful tools to investigate the apoptosis-regulatory function of IAPs.[9] It was also reported recently that antagonism of both cIAPs and XIAP is required for efficient induction of cancer cell death by IAPs antagonists.[10] On the other hand, Naito's group reported that bestatin methyl ester (MeBS: **4**, Fig. 1) specifically binds to the BIR3 domain of cIAP1 and promotes auto-ubiquitination and degradation of cIAP1.[11] Further, cIAP1 degradation induced by MeBS enhanced apoptosis of cancer cells induced by chemotherapeutic agents such as cisplastin and etoposide.[11]

Cellular retinoic acid binding protein II (CRABP-II) resides in cytoplasm and specifically binds to *all*-trans retinoic acid (ATRA: **3**, Fig. 1), an endogenous ligand of retinoic acid receptors (RARs).[12–14] CRABP-II is expressed in several cancers, including neuroblastoma and Wilms tumor.[15,16] In particular, it is thought to play a role in cancer development in MycN-amplified neuroblastoma IMR-32 cells.[13] We reported that decrease of CRABP-II induces down-regulation of MycN, activation of caspase and growth inhibition of MycN-amplified neuroblastoma IMR-32 cells.[17]

Several groups have developed methods to regulate protein levels.[18–22] We have reported protein knockdown, that is, degradation of target proteins by small molecules in living cells by making use of the E3 activity of cIAP1.[17,23–25] This approach for targeted protein degradation involves three steps; (i) formation of an artificial (non-physiological) complex of cIAP1 and a target protein via conjugation with a small molecule (named SNIPER), (ii) polyubiquitination of the target protein mediated by cIAP1's E3 activity, (iii) degradation of the polyubiquitinated protein by proteasome (Scheme 1). We previously identified compounds **1** and **2** (Fig. 1) as CRABP-II SNIPERs.[17,24] Compound **2** is a hybrid small molecule designed as a conjugate of ATRA (**3**) with MeBS (**4**). Therefore, compound **2** induces degradation of both cIAP1 and CRABP-II. On the other hand, the amide derivative of MeBS binds to cIAP1 but does not induce auto-degradation of cIAP1, and therefore amide **1** induces selective degradation of CRABP-II. The previous study also showed that degradation of both CRABP-II and cIAP1 (with **2**) results in more potent inhibition of neuroblastoma IMR32 cell proliferation than down-regulation of CRABP-II alone (with **1**). This result indicates that **2** might be a candidate therapeutic agent for MycN-amplified neuroblastoma. However, compound **2** has some issues, that is, rather low activity and chemical instability due to

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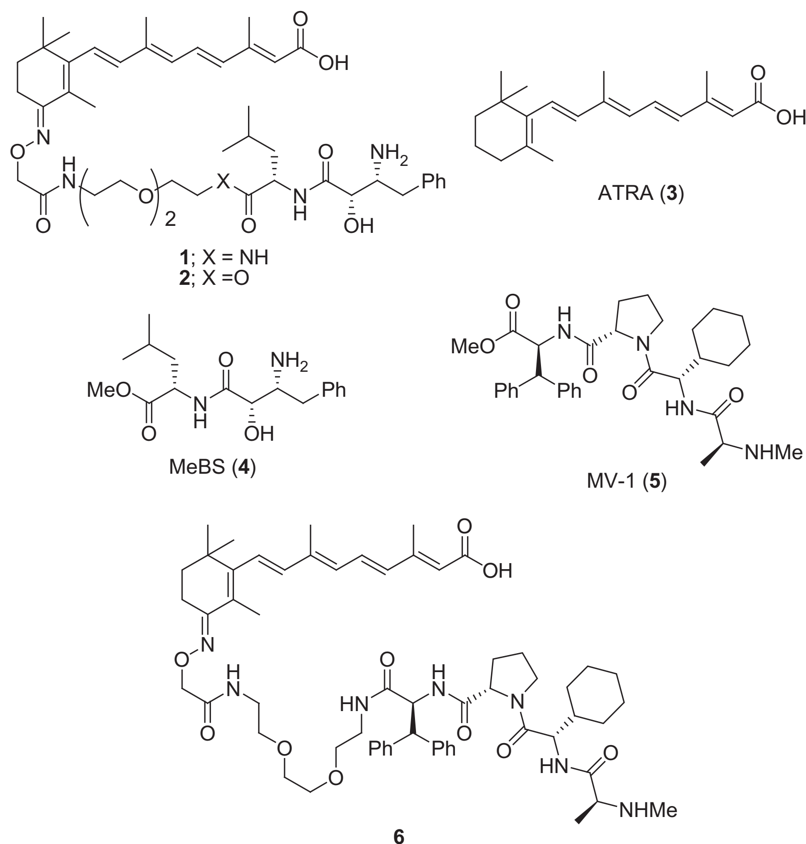
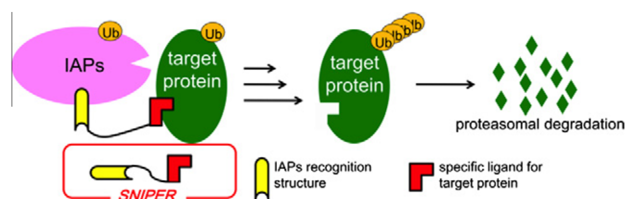


Figure 1. Structures of compound (**1**, **2**, **6**), ATRA (**3**), MeBS (**4**), and MV1 (**5**).



Scheme 1. Protein knockdown by SNIPERs.

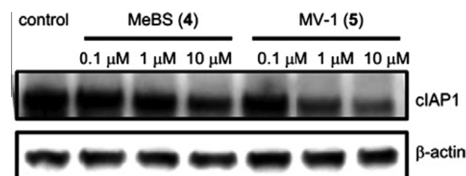


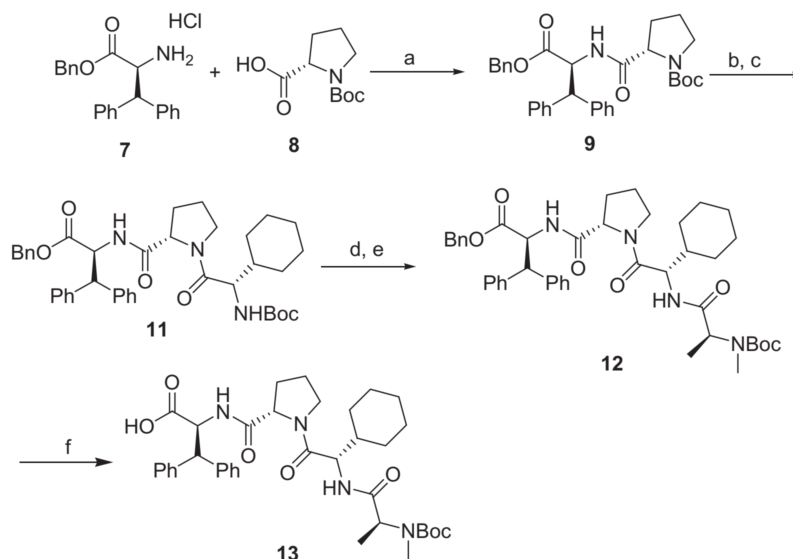
Figure 2. Western blotting detection of cIAP1 levels in HT1080 cells after 6 h treatment with compounds.

the ester group. Therefore we attempted to identify new compounds with potent activity to induce degradation of both cIAP1 and CRABP-II. Herein we describe the design, synthesis and biological evaluation of a new type of SNIPER that induces potent degradation of both cIAP1 and CRABP-II. We also investigated the effect of this SNIPER on neuroblastoma IMR-32 cells.

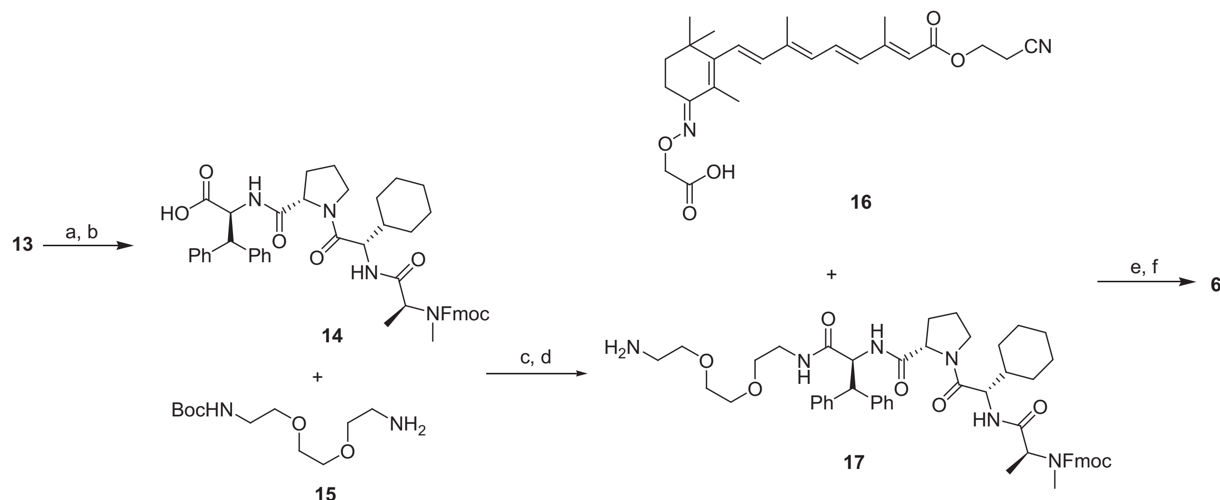
We expected that simultaneous utilization of multiple ubiquitin ligases would result in more efficient dual SNIPERs than **2**, which has a MeBS (**4**: a cIAP1-specific ligand) moiety. This consideration led us replace the MeBS (**4**) moiety with a MV1 (**5**) moiety which is a pan-antagonist for all of XIAP, cIAP1 and cIAP2.[26] MV1 (**5**) has been reported to induce cancer cell death, and is cell-permeable[26] Indeed, our studies suggested that MV1 (**5**) induces degradation of cIAP1 more effectively than does MeBS (**4**) (Fig. 2). Thus, compound **6** was designed (without an ester group; see Fig. 1). Considering the previous finding that a cIAP1/cIAP2/XIAP antagonist induces cancer cell death more potently than a cIAP1/cIAP2 antagonist,[10] we hoped that IAPs antagonism by MV1-conjugated SNIPER **6** might result in potent induction of cancer cell death. In other words, we expected that replacement of the MeBS (**4**) moiety of compound **2** with a MV1 (**5**) moiety would yield a more effective SNIPER (i.e., **6**) than **2** as a candidate for MycN-amplified neuroblastoma therapy. The linker was designed to be introduced at the C-terminus of MV1 (**5**), because (i) X-ray crystal structure determination of the complex of BIR3 domain of IAPs and AVPI tetrapeptide indicated that the C-terminus of the peptide is located outside the binding site,[27,28] and (ii) a dimer of MV1, linked at the C-terminus, showed higher binding affinities for cIAP1 and XIAP than did MV1 monomer (**5**).[26]

Synthesis of compound **6** was accomplished as shown in Schemes 2 and 3. Amine **7**,[29,30] and acid **10**[31] were prepared by using the reported procedure. Stepwise liquid-phase peptide synthesis was performed to give tetrapeptide **12**. Removal of the C-terminal *O*-benzyl group by catalytic reduction gave acid **13** (Scheme 2). Two-step conversion of the *N*-Boc group of **13** to a *N*-Fmoc group gave compound **14**. Condensation of **14** with amine **15**[17] followed by deprotection of the Boc group of the amide gave amine **17**. The condensation product of amine **17** with acid **16**, whose Fmoc group and 2-cyanoethyl group were deprotected with tetrabutylammonium fluoride (TBAF), gave compound **6**.[17,32,33]

First, we examined the cIAP1 and CRABP-II degradation-inducing activity of compound **6** in HT1080 cells overexpressing



Scheme 2. (a) 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), 1-hydroxybenzotriazole hydrate ($HOBt{\cdot}H_2O$), i-Pr_2NEt, *N,N*-dimethylformamide (DMF), rt, 22 h, 84%; (b) HCl, 1,4-dioxane, rt, 4.5 h; (c) (S)-2-(*tert*-butoxycarbonylamino)-2-cyclohexylacetic acid (**10**), EDCI, $HOBt{\cdot}H_2O$, i-Pr_2NEt, DMF, rt, 13 h, 86% (2 steps); (d) HCl, 1,4-dioxane, rt, 4.5 h; (e) (*S*)-2-[(*tert*-butoxycarbonyl)(methyl)amino]propanoic acid, EDCI, $HOBt{\cdot}H_2O$, i-Pr_2NEt, DMF, rt, 15 h, 80% (two steps); (f) Pd/C, H_2, 1,4-dioxane, rt, 6.5 h, 100%.



Scheme 3. (a) TFA, CH_2Cl_2, rt, 3.5 h, 87%; (b) 9-fluorenylmethyloxycarbonyl chloride (FmocCl), K_2CO_3, THF, rt, 3 h, 56%; (c) EDCI, $HOBt{\cdot}H_2O$, CH_2Cl_2, rt, 20 h; (d) HCl, 1,4-dioxane, rt, 1 h; (e) EDCI, $HOBt{\cdot}H_2O$, Et_3N, CH_2Cl_2, rt, 20 h; (f) tetrabutylammonium fluoride (TBAF), MeOH, THF, rt, 0.5 h, 14% (four steps; from **16**).

FLAG-cIAP1 by means of Western blotting analysis, as previously reported.[17] As expected, compound **6** induced down-regulation of both cIAP1 and CRABP-II in a dose-dependent manner (Fig. 3). Compound **6** is approximately 10 times more potent than compound **2** (Fig. 3). We previously reported that compound **1** does not bind to RARs and does not induce RARα degradation.[24] It is expected that **6** would not induce RARα degradation because both **6** and **2** possess same CRABP-recognition moiety. Next, we investigated the mechanistic features of the cIAP1/CRABP-II decrease induced by compound **6**. To confirm the contribution the ubiquitin-proteasome pathway, the incubation mixture was pretreated with proteasome inhibitor MG132 (Fig. 4). As expected, the decrease in both cIAP1 and CRABP-II levels by compound **6** was blocked by MG132. These results suggested that the reduction in cIAP1 and CRABP-II levels by compound **6** was caused by proteasomal degradation. In addition, we confirmed that the decrease in CRABP-II was not caused by a partial structure of compound **6**, or by a mere mixture of MV1 (**5**) and ATRA (**3**) (Fig. 5). Compound **6** induced a decrease of CRABP-II, whereas the mixture of MV1 (**5**) and ATRA (**3**) did not. Treatments with 10 μM MV1 (**5**), and the combination of 10 μM MV1 (**5**) and 10 μM ATRA (**3**) decreased the cIAP1 level, but did not affect the CRABP-II level in HT1080. ATRA (**3**) at 10 μM did not cause any decrease of cIAP1 or CRABP-II. Thus, conjugation of MV1 (**5**) and ATRA (**3**) in a single

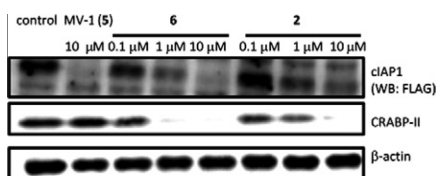


Figure 3. Down-regulation of cIAP1 and CRABP-II by treatment with compound **6**. Western blot detection of cIAP1 and CRABP-II levels in HT1080 cells expressing FLAG-tagged cIAP1 after 6 h treatment.

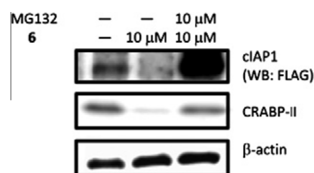


Figure 4. Influence of pretreatment with proteasome inhibitors on CRABP-II degradation induction. Western blot detection of CRABP-II levels in HT1080 cells. The cells were treated with 10 μM **6** for 6 h. MG132 (10 μM) was added to the culture 30 min prior to the addition of **6**.

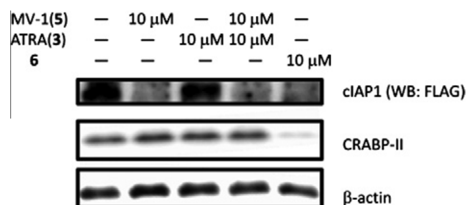


Figure 5. The influence of combination of MV-1 (**5**) and ATRA (**3**). Western blot detection of CRABP-II and cIAP1 levels in HT1080 cells expressing FLAG-tagged cIAP1 after 6-h treatment with each reagent.

molecule is mandatory for the cIAP1/CRABP-II dual degradation-inducing activity. Taken together, these results indicate that compound **6** induces cIAP1/CRABP-II dual degradation via the expected mechanism (Scheme 1). In addition, our findings indicate that protein knockdown using MV1 (**5**)-bearing molecules might be available as a general strategy by replacing the ATRA (**3**) moiety with other specific ligands of various target proteins.

Finally, we investigated the effect of compound **6** on MycN-amplified neuroblastoma IMR-32 cells. Down-regulation of CRABP-II and cIAP1 levels in IMR32 cells by **6** is shown in Figure 6. The cIAP1 and CRABP-II degradation-inducing activity decreased in the order of **6** > **2** > **1** and **6** >**1** = **2**, respectively (Fig. 6). We also evaluated the cell proliferation-inhibiting activity of compounds **6** and **2**. Compound **6** showed strong inhibition of cell proliferation, and the activity was higher than that of compound **2** (Fig. 7). Then, we tested caspase 3/7 activation by compound **6** (Table 1). Compound **1**, which is a weaker inhibitor of IMR-32 cell proliferation than **2**,[17] enhanced caspase activity about 1.8-fold compared with the controls. The caspase activity enhancement elicited by compound **2** was estimated to be about 3.6-fold. Compound **6** showed the most potent caspase activity enhancement among the tested compounds, that is about 4.4-fold. These results are consistent with the idea that **6** inhibits IMR32 cell proliferation by decreasing CRABP-II and cIAP1, thereby resulting in activation of caspase 3/7.

In summary, we have prepared a novel SNIPER molecule, compound **6**, in which ATRA (**3**) and MV1 (**5**) moieties are conjugated via a linker. Compound **6** induced efficient degradation of both cIAP1 and CRABP-II. Mechanistic analysis indicated that cIAP1 and CRABP-II were degraded in a proteasome-dependent manner. Compound **6** also showed stronger cell proliferation-inhibiting and caspase-activating activities than **2** in the assay using MycN-amplified neuroblastoma IMR-32 cells. These results suggested that compound **6** might be a new therapeutic candidate/lead compound for the treatment of neuroblastoma. Dual degradation of cancer-related proteins, including IAPs, by treatment with IAPs antagonist-bearing molecules seems likely to be of therapeutic value as a novel strategy for cancer therapy. A detailed mechanistic analysis of cancer cell death induced by **6** is under way.

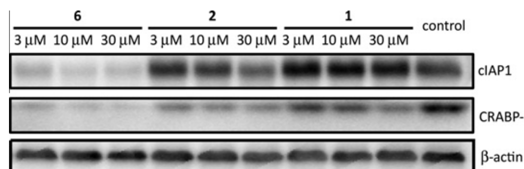


Figure 6. Western blot detection of cIAP1 and CRABP-II levels in IMR32 cells after 24 h treatment.

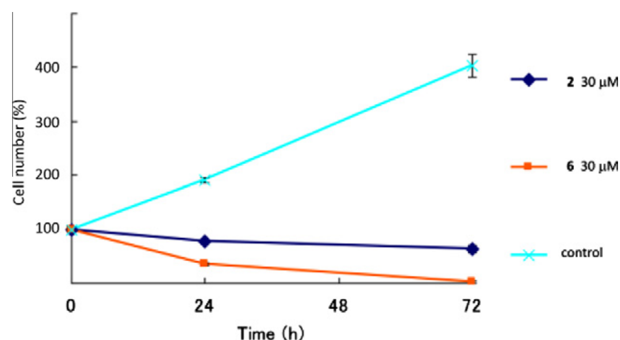


Figure 7. Relative cell numbers of IMR-32 cells treated with **2** and **6**.

Table 1
Effects of compounds on caspase activity[a,b]

Entry	Compound	Caspase activity (fold increase)
1	–	1.00
2	**1**	1.84
3	**2**	3.61
4	**6**	4.44

[a] Values are means of at least two experiments.
[b] IMR-32 cells were treated with compounds for 24 h. The data are the means of triplicate determinations.

Acknowledgments

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