



## Development of target protein-selective degradation inducer for protein knockdown

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### ABSTRACT

Our previous technique for inducing selective degradation of target proteins with ester-type SNIPER (Specific and Nongenetic Inhibitor-of-apoptosis-proteins (IAPs)-dependent Protein ERaser) degrades both the target proteins and IAPs. Here, we designed a small-molecular amide-type SNIPER to overcome this issue. As proof of concept, we synthesized and biologically evaluated an amide-type SNIPER which induces selective degradation of cellular retinoic acid binding protein II (CRABP-II), but not IAPs. Such small-molecular, amide-type SNIPERs that induce target protein-selective degradation without affecting IAPs should be effective tools to study the biological roles of target proteins in living cells.

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## 1. Introduction

Methods to down-regulate target proteins in cells or animals are useful not only for biological studies and medical research, but also for developing therapeutic strategies in cases where expression of a target protein is involved in the pathogenesis of disease. Thus far, deletion and suppression of proteins at the pre-translational level by means of gene knockout and gene knock-down have been widely used for ablating target proteins. Such techniques have yielded insight into the biological functions of numerous proteins in cells or animals. However, complicated and time-consuming genetic manipulation is required for gene knock-out. Gene knockdown using RNA interference is easy, but cannot remove existing proteins and so is especially ineffective for proteins with a long half-life. Therefore, the utility of these genetic methods for medical applications is limited. Proteolysis-targeting chimeric molecules (protacs) represent a pioneering approach in the field of down-regulating proteins post-translationally.<sup>1</sup> However, the membrane permeability or stability of peptide structures is often inadequate for biological studies and therapeutic applications.<sup>2</sup> Therefore, new general techniques, which overcome these issues, are needed.

Recently, we have reported protein knockdown as a technique for inducing selective degradation of proteins post-translationally by using small molecules which have sufficient membrane permeability.<sup>3</sup> This approach mimics physiological caspase degradation by inhibitor of apoptosis proteins (IAPs), which show ubiquitin

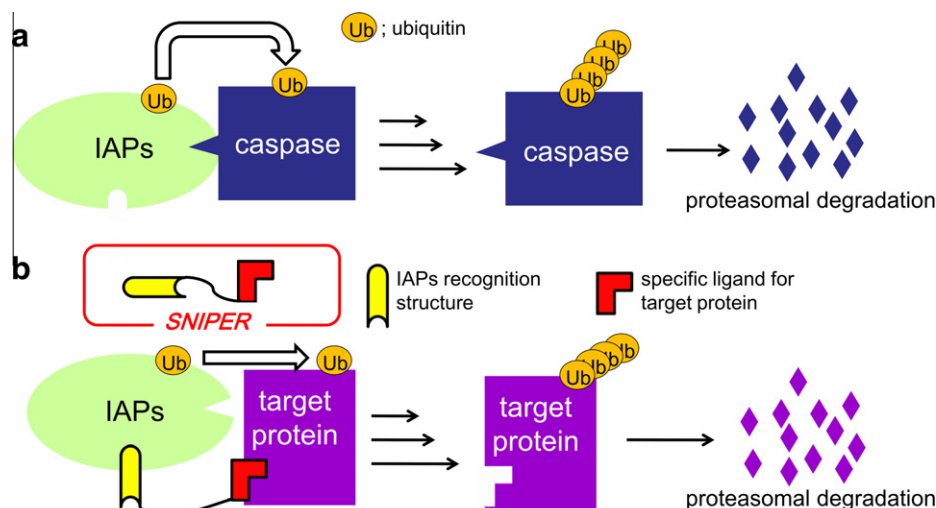
ligase (E3) activity.<sup>4,5</sup> Under physiological conditions, caspase degradation occurs via two steps: (i) IAPs bind to caspases and promote poly-ubiquitination, (ii) the polyubiquitinated caspases are degraded by proteasome (Fig. 1a). Protein knockdown is achieved by the use of small molecules designed as conjugates of an IAP recognition moiety with a specific ligand for a target protein. Such a molecule, which we named SNIPER (Specific and Nongenetic IAPs-dependent Protein ERaser), induces formation of an artificial (non-physiological) complex of IAP and the target protein, thereby promoting poly-ubiquitination and proteasomal degradation of the target protein (Fig. 1b). We adopted methyl bestatin (MeBS, **2**) (Fig. 1)<sup>6,7</sup> as a ligand for cellular inhibitor of apoptosis protein 1 (cIAP1), which is one of the IAPs,<sup>5,8</sup> and all-*trans* retinoic acid (ATRA, **3**) (Fig. 2) as a specific ligand for cellular retinoic acid binding protein II (CRABP-II),<sup>9</sup> and synthesized SNIPER (**4**) (Fig. 3)<sup>3</sup> as a tool for degradation of CRABP-II. However, this ester-type SNIPER (**4**) was found to promote auto-degradation of cIAP1, like MeBS (**2**),<sup>3,6,7</sup> and thus, the selectivity of ester-type SNIPER (**4**) for its target protein is poor. In addition, the ester group of **4** might be easily hydrolyzed in cells. Therefore, we aimed to develop a new type of SNIPER which would overcome these problems. Herein, we describe the design, synthesis and biological evaluation of an amide-type SNIPER which induces selective degradation of CRABP-II, without affecting IAPs.

## 2. Chemistry

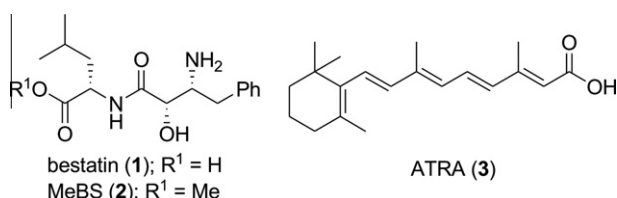
The compounds prepared for this study are shown in Figure 3 (compounds **5–8**) and Figure 6 (DanBE, **16**). Syntheses were carried

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**Figure 1.** (a) Physiological protein degradation. (b) Artificial protein degradation (protein knockdown) induced by SNIPER.



**Figure 2.** Structures of bestatin (1), bestatin methyl ester (MeBS, 2), and ATRA (3).

out as outlined in Schemes 1–3. The route for synthesis of compounds 5 and 7 is illustrated in Scheme 1. Condensation of amine 10 with acid 11 afforded amide 12. Hydrolysis of ester 12 under an alkaline condition gave acid 14. Bestatin (1) was treated with di-*tert*-butyl dicarbonate ((Boc)<sub>2</sub>O) to yield *N*-Boc bestatin (13). Condensation of acid 13 or 14 with methylamine afforded amides 15 and 7. Removal of the Boc group of 15 gave BE04 (5).

DanBE (16) was synthesized from 17 and 18 via the route shown in Scheme 2. Condensation of sulfonyl chloride 17 with amine 18 gave sulfonamide 19. Alcohol 19 was allowed to react

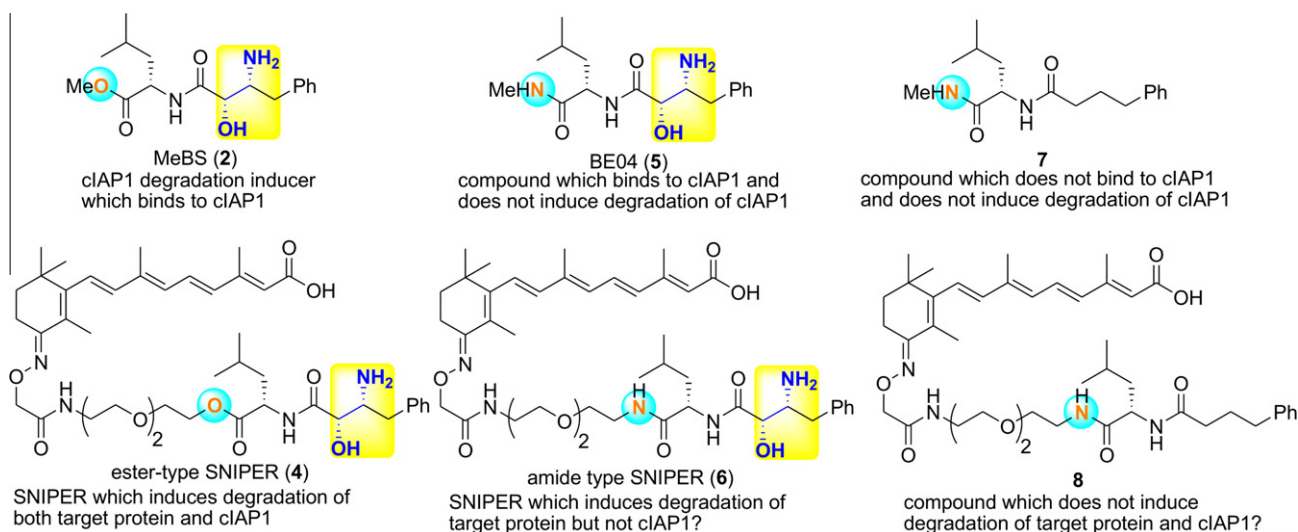
with *N*-Boc bestatin (14) and subsequent deprotection of the Boc group of compound 20 gave DanBE (16).

Compounds 6 and 8 were synthesized as outlined in Scheme 3. Alcohol 21<sup>10</sup> was converted to azide 23 by tosylation and subsequent treatment with sodium azide. Amine 24, obtained by reduction of azide 23, was allowed to react with compounds 14 and 25<sup>3</sup> to yield amides 26 and 27. Condensation of acid 28<sup>3</sup> with amines 29 and 30, derived by deprotection of the Boc group of compounds 26 and 27, afforded amides 31 and 32. Deprotection of the 2-cyanoethyl groups of compounds 31 and 32 with tetrabutylammonium fluoride (TBAF) gave acid 33 and compound 8.<sup>3,11</sup> Removal of the 9-fluorenylmethyloxycarbonyl (Fmoc) group of compound 33 with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)<sup>3,12</sup> gave compound 6.

### 3. Results and discussion

#### 3.1. Molecular design

We selected MeBS (2) as a ligand for cIAP1 in the previous study because MeBS (2) activates auto-ubiquitination of cIAP1 and



**Figure 3.** Structures and molecular design of SNIPERs.

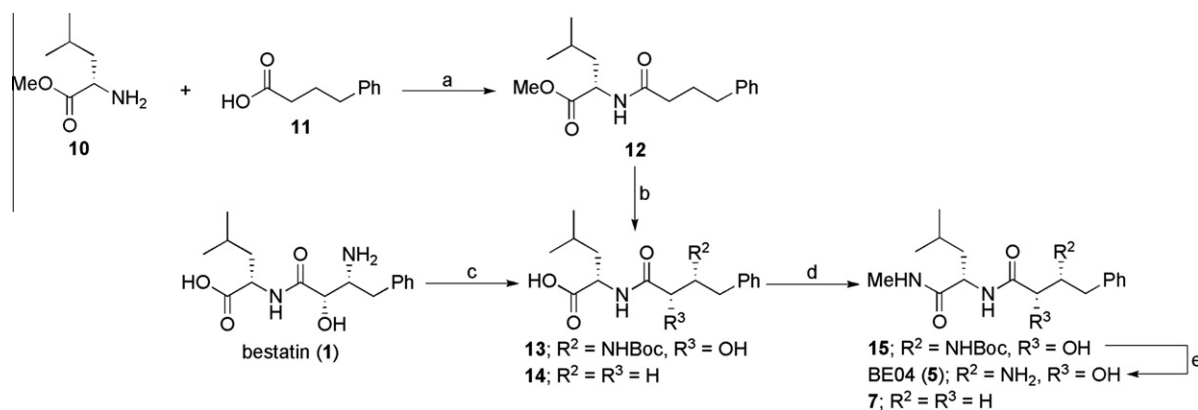
maintains the E3 activity of cIAP1. Therefore, we considered that highly selective SNIPERs might be obtained by replacement of MeBS (**2**) in the ester-type SNIPER (**4**) with a structure which binds to cIAP1, but does not induce auto-degradation of cIAP1 and does not inhibit the E3 activity of cIAP1. Therefore we attempted to identify such a structure. Naito's group investigated the cIAP1-binding affinity and auto-degradation activity of MeBS analogs.<sup>6</sup> The order of binding affinity evaluated by means of surface plasmon resonance (SPR) binding experiments indicated that the binding affinity of BE04 (**5**) (Fig. 3) is higher than that of bestatin (**1**), which induced degradation of cIAP1, but lower than that of MeBS (**2**). On the other hand, the cIAP1-degradation activity of BE04 (**5**) is apparently weaker than that of bestatin (**1**) or MeBS (**2**).<sup>6</sup> These results indicate that the two activities (binding activity and auto-degradation activity) should be separable. We further investigated the effect of BE04 (**5**) on cIAP1 levels by means of Western blotting. Treatment of HT1080 cells expressing FLAG-tagged cIAP1 with even 1000  $\mu$ M BE04 (**5**) had no influence on cIAP1 levels (Fig. 4a–c). Accordingly, it appears that BE04 (**5**) does not induce auto-degradation of cIAP1. In addition, the results shown in Figure 5 indicate that BE04 (**5**) has no influence on E3 activity, because ubiquitination of cIAP1 is mainly mediated by the E3 activity of cIAP1 itself.<sup>13</sup> Next, we investigated the binding of BE04 (**5**) to cIAP1. The results of fluorescence polarization (FP) assay<sup>7</sup> indicated that BE04 (**5**) did bind to cIAP1 (Fig. 6) and showed competitive binding with respect to DanBE (**16**),<sup>7</sup> a FP probe. Moreover, it was suggested that BE04 (**5**) and MeBS (**2**) bound to cIAP1 in a mutually competitive manner by means of Western blot analysis (Fig. 4b). Taken together, these results indicate that BE04 (**5**) has the activity we expected. Therefore, it appears that substitution of the ester group of **4** to amide greatly decreases cIAP1-degradation activity, but the binding affinity is

partially retained and the amide has no influence on E3 activity. This substitution is also expected to be advantageous for prolonging the duration of target protein destabilization, because amide groups are generally more stable than ester groups in the intracellular environment. Based on these findings, we designed and synthesized SNIPER (**6**) as a selective degradation inducer of CRABP (Fig. 3). We also prepared compound **8** as a negative control (Fig. 3), based on compound **7**,<sup>14</sup> which does not bind to cIAP1 and does not induce auto-degradation of cIAP1 (Figs. 4a, c and 6).

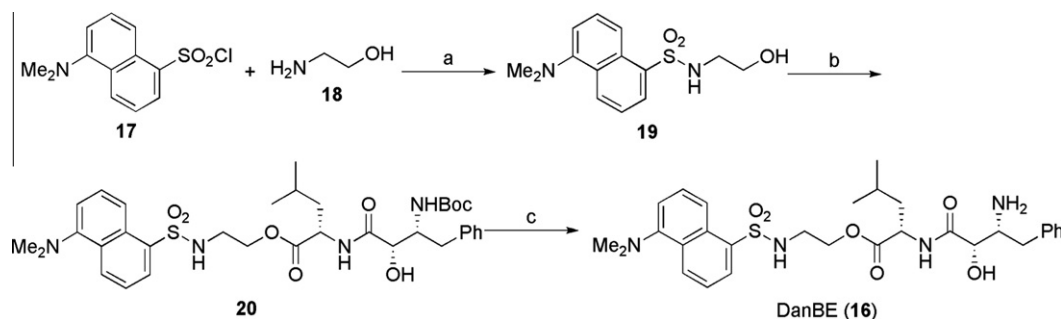
### 3.2. Selectivity and time course of degradation of CRABP-II by the synthesized compound

We initially evaluated the cIAP1/CRABP-II degradation selectivity of SNIPER (**6**) and compound **8**. The CRABP-II degradation activity of **6** was similar to that of SNIPER (**4**) as evaluated by Western blot analysis. As expected, **6** had no influence on cIAP1 levels even at 30  $\mu$ M (Fig. 7a and b). On the other hand, compound **8** did not induce degradation of either cIAP1 or CRABP-II (Fig. 7a). Next we examined whether **6** induced degradation of X-linked inhibitor of apoptosis protein (XIAP) and cellular inhibitor of apoptosis protein 2 (cIAP2), which (i) are members of the IAP family, (ii) have E3 activity, and (iii) induce auto-degradation.<sup>5,8</sup> As shown in Figs. 4b, c, and 6 had no effect on the levels of XIAP and cIAP2, as was the case for BE04 (**5**) (Fig. 7c and d). These results indicated that amide-type SNIPER (**6**) had high specificity for degradation of CRABP-II. In addition, the reduced CRABP-II level was maintained for 12 h with **4**, whereas it was maintained for 48 h with **6** (Fig. 7e). The replacement of the ester group with amide thus greatly prolonged the duration of CRABP-II destabilization.

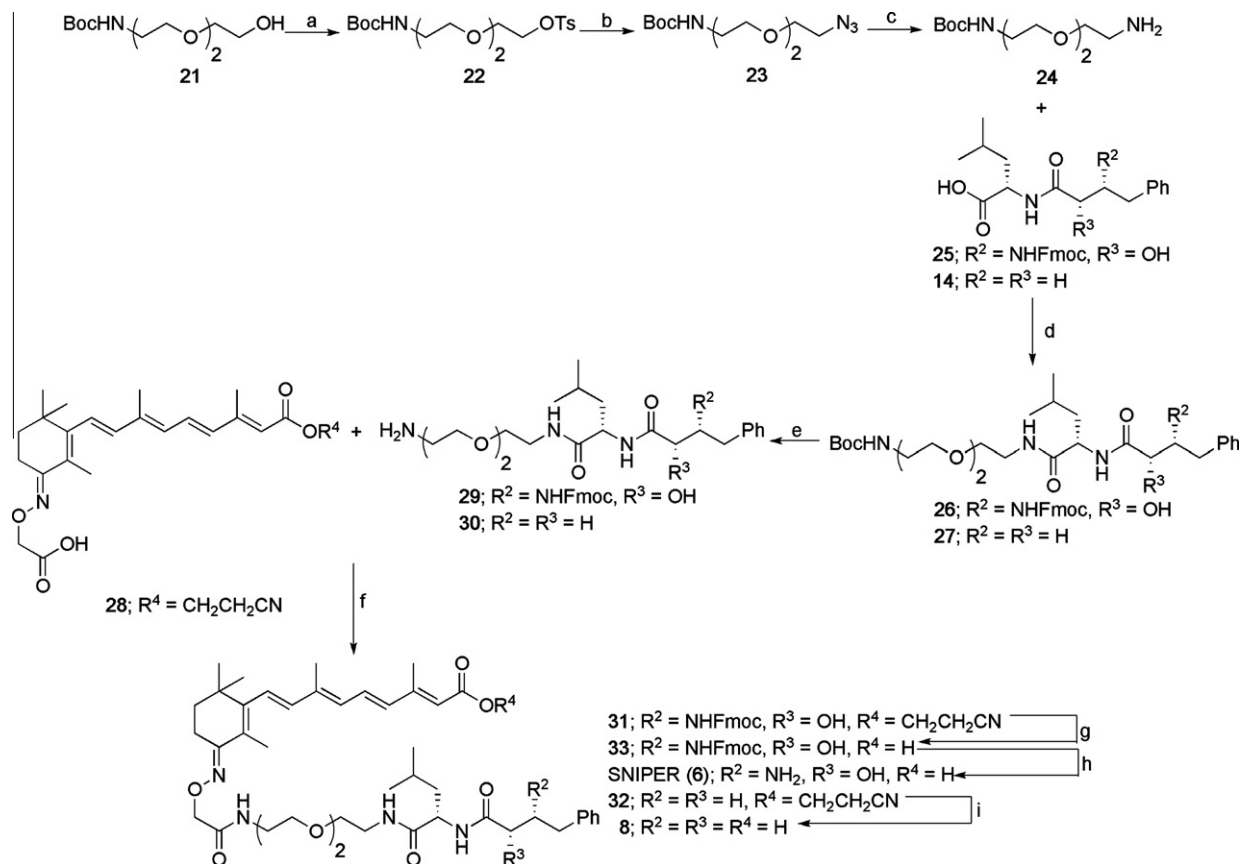
We next confirmed the mechanism of CRABP-II degradation by **6**, using the previously reported methods,<sup>3</sup> including pretreatment



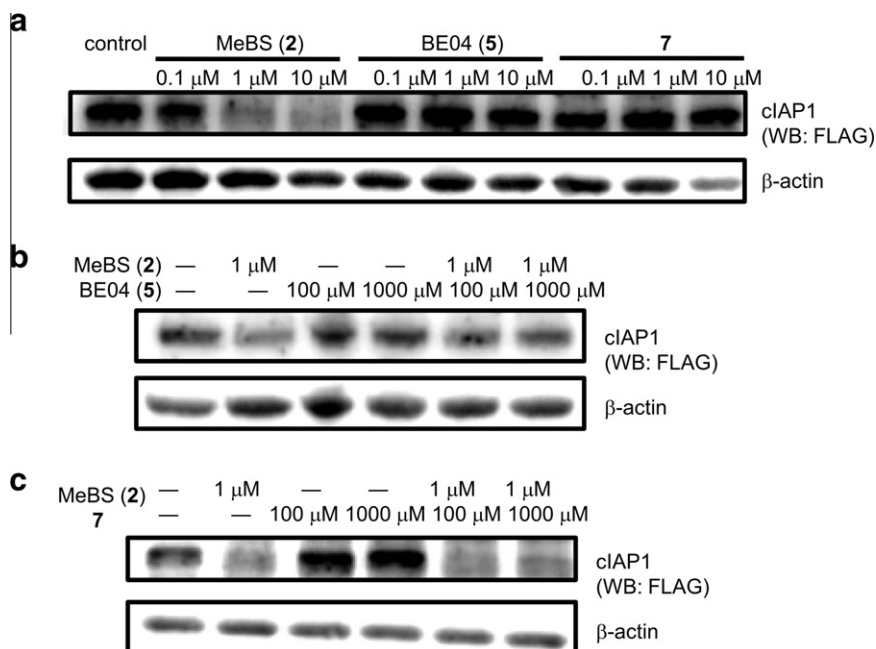
**Scheme 1.** Reagents and conditions: (a) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI), 1-hydroxybenzotriazole hydrate (HOBt·H<sub>2</sub>O), Et<sub>3</sub>N, tetrahydrofuran (THF), rt, 100%; (b) LiOH, MeOH, H<sub>2</sub>O, rt, 93%; (c) di-*tert*-butyl dicarbonate, NaOH, H<sub>2</sub>O, acetone, rt, 61%; (d) methylamine hydrochloride, EDCI, HOBt·H<sub>2</sub>O, iPr<sub>2</sub>NEt, dimethylformamide (DMF), CH<sub>2</sub>Cl<sub>2</sub>, rt, 88–99%; (e) HCl, 1,4-dioxane, rt, 99%.



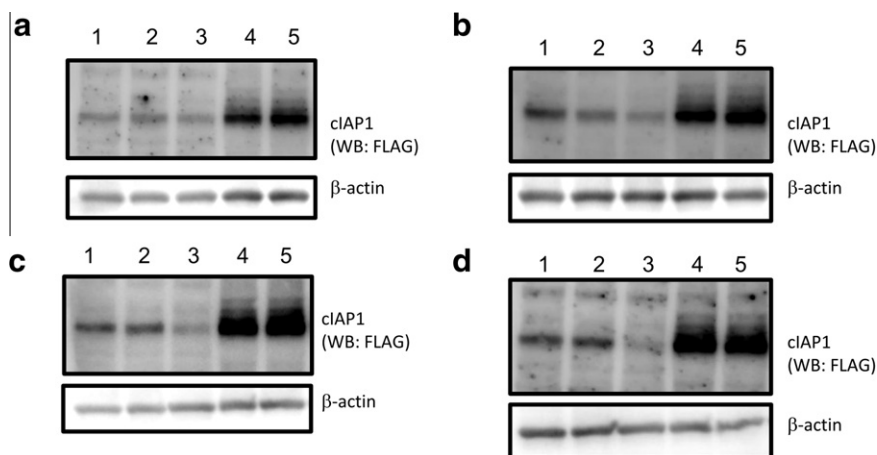
**Scheme 2.** Reagents and conditions: (a) Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>, rt, 68%; (b) *N*-Boc bestatin (**13**), EDCI, HOBt·H<sub>2</sub>O, iPr<sub>2</sub>NEt, CH<sub>2</sub>Cl<sub>2</sub>, rt, 24%; (c) HCl, 1,4-dioxane, CH<sub>2</sub>Cl<sub>2</sub>, rt, 100%.



**Scheme 3.** Reagents and conditions: (a) *p*-toluenesulfonyl chloride, *N,N*-dimethylaminopyridine (DMAP), Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>, rt; (b) NaN<sub>3</sub>, DMF, 60 °C, 98% (two steps); (c) Pd/C, H<sub>2</sub>, EtOH, rt, quant.; (d) EDCI, HOBT·H<sub>2</sub>O, CH<sub>2</sub>Cl<sub>2</sub>, rt, 78–88%; (e) HCl, 1,4-dioxane, CH<sub>2</sub>Cl<sub>2</sub>, quant.; (f) EDCI, HOBT·H<sub>2</sub>O, THF, rt, 56–62%; (g) tetrabutylammonium fluoride (TBAF), MeOH, THF, rt; (h) 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), *n*-C<sub>12</sub>H<sub>25</sub>SH, CH<sub>2</sub>Cl<sub>2</sub>, rt, 57% (two steps); (i) TBAF, MeOH, THF, rt, 70%.



**Figure 4.** (a) Western blot-detection of cIAP1 levels in HT1080 cells expressing FLAG-tagged cIAP1. The cells were treated with compounds for 6 h. (b) BE04 (5) or (c) compound 7 was added to the culture 1 h prior to the addition of MeBS (2). BE04 (5) inhibited degradation of cIAP1 by MeBS (2) and compound 7 did not. These results suggested that: (i) BE04 (5) and compound 7 do not induce degradation of cIAP1, (ii) BE04 (5) and MeBS (2) bind to cIAP1 in a mutually competitive manner in cells, but compound 7 and MeBS (2) do not show competitive binding.



**Figure 5.** Western blot-detection of cIAP1 levels in HT1080 cells expressing FLAG-tagged cIAP1 after (a) 15-min, (b) 30-min, (c) 60-min, and (d) 120-min treatment with reagents. MG132 was added to the culture 30 min prior to the addition of MeBS (**2**) or BE04 (**26**). Lane 1, control; lane 2, 1000  $\mu$ M BE04 (**26**); lane 3, 30  $\mu$ M MeBS (**2**); lane 4, 10  $\mu$ M MG132; lane 5, mixture of 10  $\mu$ M MG132 and 1000  $\mu$ M BE04 (**26**). Proteasome inhibitor MG132 inhibited auto-degradation of cIAP1 and increased the accumulation of cIAP1, but BE04 (**5**) had no influence on cIAP1 level. In addition, BE04 (**5**) had no influence on the accumulation of cIAP1 induced by MG132 (lanes 4 and 5). Given that ubiquitination and degradation of cIAP1 are mediated by the ubiquitin ligase activity of cIAP1 itself,<sup>8</sup> these results indicate that BE04 (**5**) did not inhibit the ubiquitin ligase activity of cIAP1.

with an excess of MeBS (**2**) (Fig. 8a), GST pull-down assay (Fig. 8b), combined use of proteasome inhibitors (Fig. 8c), combined use of MeBS (**2**) or BE04 (**5**) and ATRA (**3**) (Fig. 9), RAR $\alpha$  degradation assay and RAR reporter gene assay (data not shown). In these mechanistic analyses, all actions of **6** were similar to those of **4**, except for degradation activity towards cIAP1. Therefore, SNIPER (**6**) is suggested to induce (i) formation of an artificial ternary complex with cIAP1 and CRABP-II, (ii) ubiquitination of CRABP-II by cIAP1, and (iii) degradation of ubiquitinated CRABP-II by proteasome.<sup>3</sup>

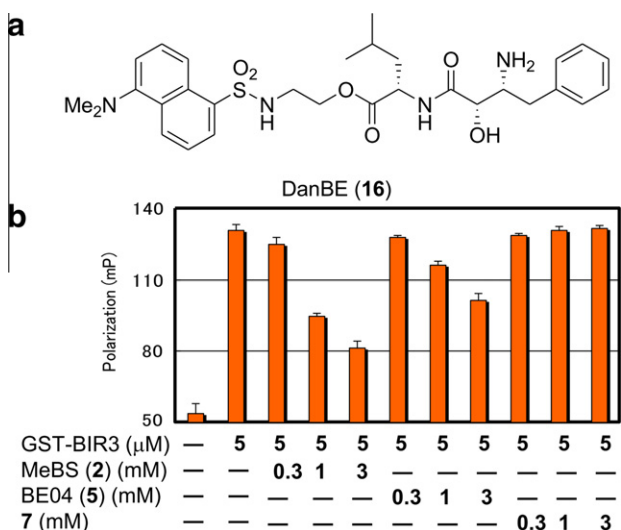
### 3.3. Effect on cIAP1 inhibition at the level of function by the synthesized compound

Although amide-type SNIPER (**6**) degraded CRABP-II without affecting cIAP1 levels, **6** may inhibit the biological function of

cIAP1, because it binds to cIAP1. Such inhibition would be unfavorable for investigation of the biological function of a target protein by protein knockdown. Therefore, we investigated whether **6** inhibits the function of cIAP1. It is thought that inhibition of IAP function activates caspase 3/7 and induces apoptosis.<sup>5,15</sup> Indeed, cIAP1 destabilization by MeBS (**2**) enhanced apoptosis of cancer cells by chemotherapeutic drugs such as cisplatin and etoposide (ETP).<sup>6</sup> Therefore, we evaluated the effect on apoptosis in HT1080 cells of MeBS (**2**), ester-type SNIPER (**4**) and **6** in the presence or absence of ETP, using lactate dehydrogenase (LDH) assay (Fig. 10a). In the absence of etoposide, MeBS (**2**), **4**, and **6** hardly induced apoptosis. However, MeBS (**2**) and **4**, which induce cIAP1 degradation, enhanced apoptosis of HT1080 cells by ETP in a dose-dependent fashion, while **6**, which has no effect on cIAP1 levels, did not enhance apoptosis of HT1080 cells. Furthermore, we investigated the effects of these compounds on caspase 3/7 activation. As shown in Figure 10b, these compounds did not activate caspase 3/7 in the absence of ETP. In the presence of ETP, MeBS (**2**) and **4** activated caspase 3/7, whereas **6** had no effect on caspase 3/7. Collectively, these results suggest that the binding of **6** to cIAP1 neither induces degradation of cIAP1 nor inhibits cIAP1 function. All the results mentioned above indicated that amide-type SNIPER (**6**) exhibits satisfactory selectivity and stability, overcoming the issues found in the case of ester-type SNIPER.

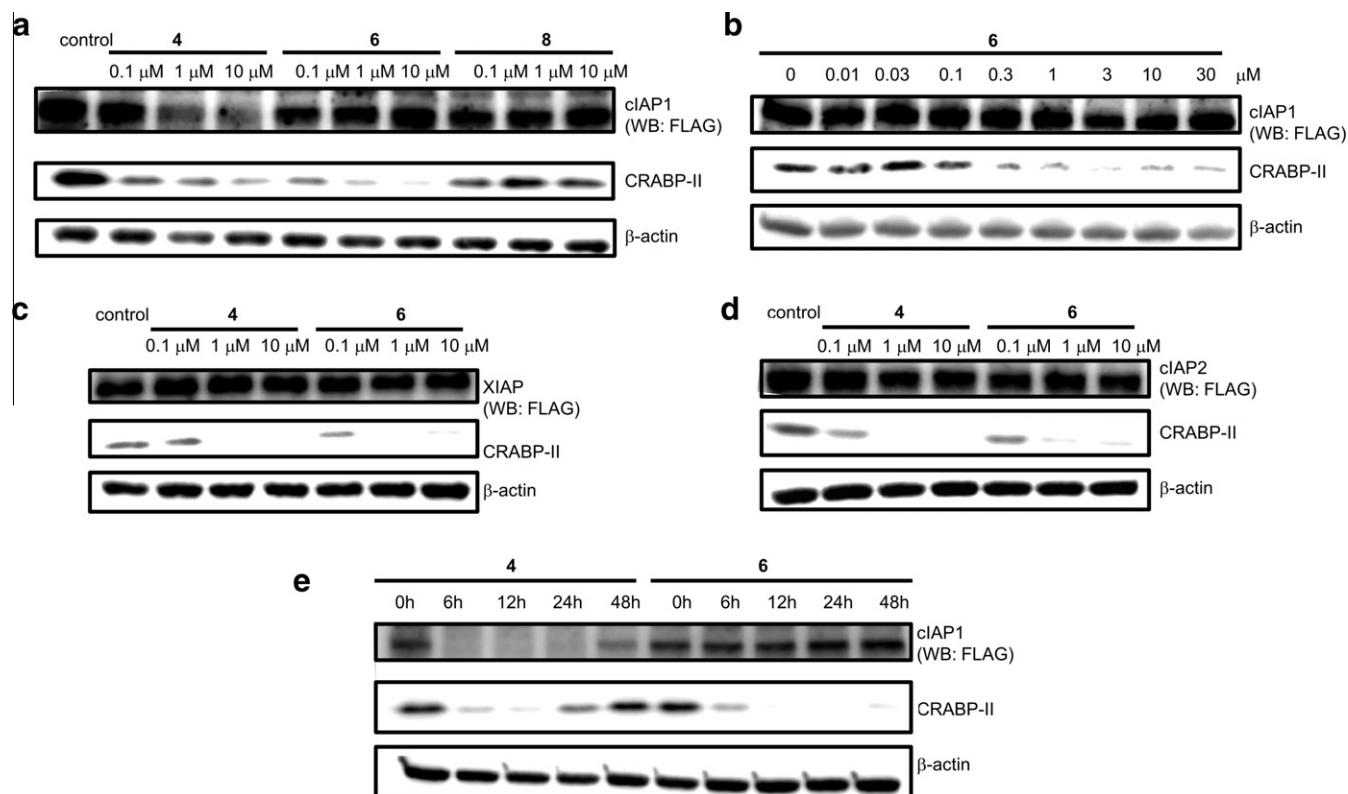
### 3.4. Characterization of CRABP-II in neuroblastoma cells using SNIPER

Finally, we attempted to demonstrate that amide-type SNIPER is useful for investigation of the biological function of CRABP-II by protein knockdown. It was reported that CRABP-II increases expression of the oncogene MycN in neuroblastoma cells.<sup>16</sup> In addition, MycN inhibits caspase and the apoptotic pathway<sup>17</sup> and MycN knockdown inhibits proliferation of neuroblastoma cells.<sup>18</sup> Therefore, we investigated the effects of amide-type SNIPER (**6**) on MycN expression, caspase activation and proliferation of neuroblastoma cells. Consistent with the reported role of CRABP-II in MycN expression, **6** decreased MycN levels (Fig. 11a) concomitantly with a decrease of CRABP-II protein (Fig. 11b). Next, we investigated the effects of MeBS (**2**), BE04 (**5**), and **6** on caspase 3/7 in IMR-32 cells (Fig. 11c). MeBS (**2**) and BE04 (**5**) hardly activated caspase 3/7, whereas **6** significantly activated caspase

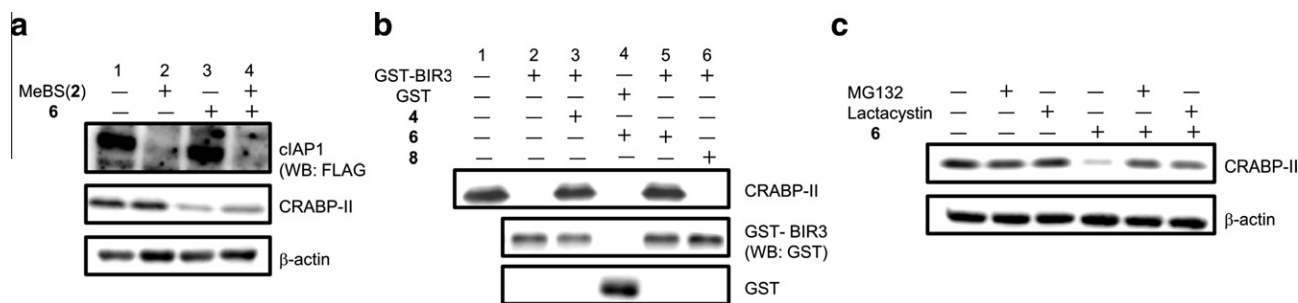


**Figure 6.** (a) Structure of DanBE (**16**), a fluorescence polarization (FP) probe. (b) Binding assay. Competitive displacement studies were performed with MeBS (**2**), BE04 (**5**), and compound **7** by means of FP assay. MeBS (**2**), BE04 (**5**) or compound **7** was incubated in the presence of DanBE (**16**) and GST-BIR-3 (GST-tagged BIR3 domain of cIAP1, to which MeBS (**2**) binds) for 5 min at room temperature in phosphate-buffered saline (pH 7.4). After incubation, the fluorescence polarization of DanBE (**16**) was measured.





**Figure 7.** (a) Western blot-detection of CRABP-II levels in HT1080 cells expressing FLAG-tagged cIAP1 after 6-h treatment with compounds **4**, **6**, and **8**. (b) Western blot-detection of CRABP-II and cIAP1 levels in HT1080 cells expressing FLAG-tagged cIAP1 after 6-h treatment with SNIPER (**6**). Compound **6** induced dose-dependent down-regulation of CRABP-II, but not cIAP1. (c) Western blot-detection of XIAP levels in HT1080 cells expressing FLAG-tagged XIAP. The cells were treated with compounds for 6 h. (d) Western blot-detection of cIAP2 levels in HT1080 cells expressing FLAG-tagged cIAP2. The cells were treated with compounds for 6 h. (e) Western blot-detection of cIAP1 levels and CRABP-II in HT1080 cells expressing FLAG-tagged cIAP1 treated with ester-type SNIPER (**4**) and amide-type SNIPER (**6**). With **4**, the reduced CRABP-II level was maintained for 12 h whereas with **6**, it was maintained for 48 h.

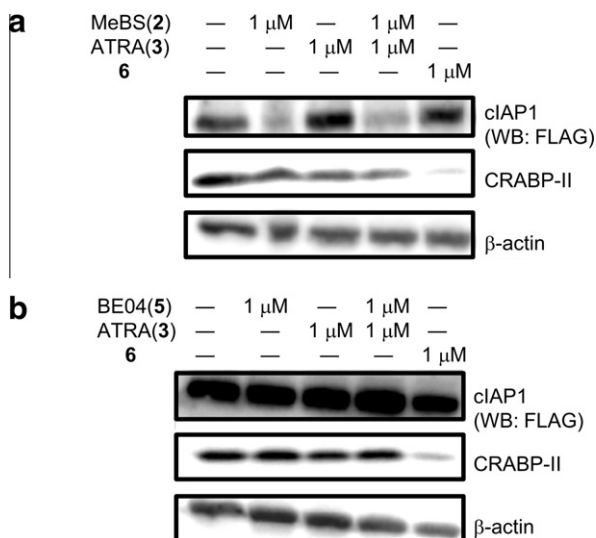


**Figure 8.** (a) Influence of pretreatment of HT1080 cells expressing FLAG-tagged cIAP1 with MeBS (**2**) on induction of CRABP-II degradation. The cells were treated with 1 μM **6** for 6 h. MeBS (**2**) (1000 μM) was added to the culture 1 h prior to the addition of **6**. Pretreatment with an excess amount of MeBS (**2**) led to the complete disappearance of cIAP1 (lane 2). CRABP-II levels were down-regulated by treatment with amide-type SNIPER (**6**) [without pretreatment with MeBS (**2**)] as mentioned above (lane 3), whereas amide-type SNIPER (**6**) scarcely decreased the CRABP-II levels in cells pretreated with 1000 μM MeBS (**2**) (lane 4). These results indicate that the reduction of CRABP-II by amide-type SNIPER (**6**) is mediated by cIAP1. (b) Western blot-detection of GST-BIR3, GST, and CRABP-II levels of samples prepared by pull-down assay in vitro: lane 1, CRABP-II levels; lane 2, mixture of GST-BIR3 and CRABP-II; lane 3, mixture of GST-BIR3, CRABP-II, and **4**; lane 4, mixture of GST, CRABP-II, and **6**; lane 5, mixture of GST-BIR3, CRABP-II, and **6**; lane 6, mixture of GST-BIR3, CRABP-II, and **8**. GST-BIR3 pulled down CRABP-II in the presence of amide-type SNIPER (**6**) (lane 5) as well as ester-type SNIPER (**4**) (lane 3), but not in the absence of amide-type SNIPER (**6**) (lane 2) or in the presence of compound **8** (lane 6). GST did not co-precipitate CRABP-II even in the presence of amide-type SNIPER (**6**) (lane 4). This result indicates that CRABP-II is held in proximity to cIAP1 by amide-type SNIPER (**6**), but not by compound **8**. (c) Influence of pretreatment with proteasome inhibitors on induction of CRABP-II degradation. Western blot-detection of CRABP-II level in HT1080 cells expressing FLAG-tagged cIAP1. The cells were treated with 1 μM **6** for 6 h. MG132 (10 μM) and lactacystin (10 μg/mL) were added to the culture 30 min prior to the addition of **6**. The down-regulation of CRABP-II by amide-type SNIPER (**6**) was cancelled by proteasome inhibitors, MG132 and lactacystin. This result suggested that the down-regulation of CRABP-II induced by amide-type SNIPER (**6**) is mediated by proteasomal degradation.

3/7. Finally, we evaluated inhibition of cell proliferation by MeBS (**2**), BE04 (**5**), and **6**, using IMR-32 cells (Fig. 12). The results were consistent with the results of caspase 3/7 assay. MeBS (**2**) and BE04 (**5**) did not show strong inhibition of cell proliferation, whereas **6** showed strong and dose-dependent inhibition. On the other hand, **6** hardly inhibited proliferation of HT1080 and

MCF-7 cells, which express CRABP-II and cIAP1, but not MycN, though it decreased CRABP-II in these cells (Fig. 13). These results suggested that the cell proliferation inhibition by amide-type SNIPER (**6**) is specific for neuroblastoma cells expressing MycN.

With regard to caspase activation and cell proliferation, ester-type SNIPER (**4**), as well as the combination of amide-type SNIPER



**Figure 9.** (a) Influence of combination of MeBS (2) and ATRA (3). Western blot-detection of CRABP-II and cIAP1 levels in HT1080 cells expressing FLAG-tagged cIAP1. The cells were treated with 1 μM MeBS (2), 1 μM ATRA (3), or 1 μM 6 for 6 h. (b) Influence of combination of BE04 (5) and ATRA (3). Western blot-detection of CRABP-II and cIAP1 levels in HT1080 cells expressing FLAG-tagged cIAP1. The cells were treated with 1 μM BE04 (2), 1 μM ATRA (3), or 1 μM 6 for 6 h. SNIPER (6) induced a decrease of CRABP-II, whereas the mixture of MeBS (2) or BE04 (5) and ATRA (3) did not. The treatments with MeBS (2) or BE04 (5), and the combination of MeBS (2) or BE04 (5) and ATRA (3) did not affect the CRABP-II level in HT1080. ATRA (3) did not cause any decrease of cIAP1 or CRABP-II. Thus, single treatment of HT1080 cells expressing FLAG-tagged cIAP1 with BE04 (5) and ATRA (3), which are partial structures of SNIPER (6), or their combination, did not induce degradation of CRABP-II.

(6) and MeBS (2), showed a stronger effect than amide-type SNIPER (6) alone (Figs. 11c and 14), even though 4 and 6 equally decreased CRABP-II and MycN (Fig. 11a and b). These results suggested that down-regulation of both cIAP1 and CRABP-II is more effective than down-regulation of CRABP-II alone to inhibit cell growth and induce apoptosis of neuroblastoma cells. This discovery was made possible by the development of CRABP-II-specific amide-type SNIPER (6).

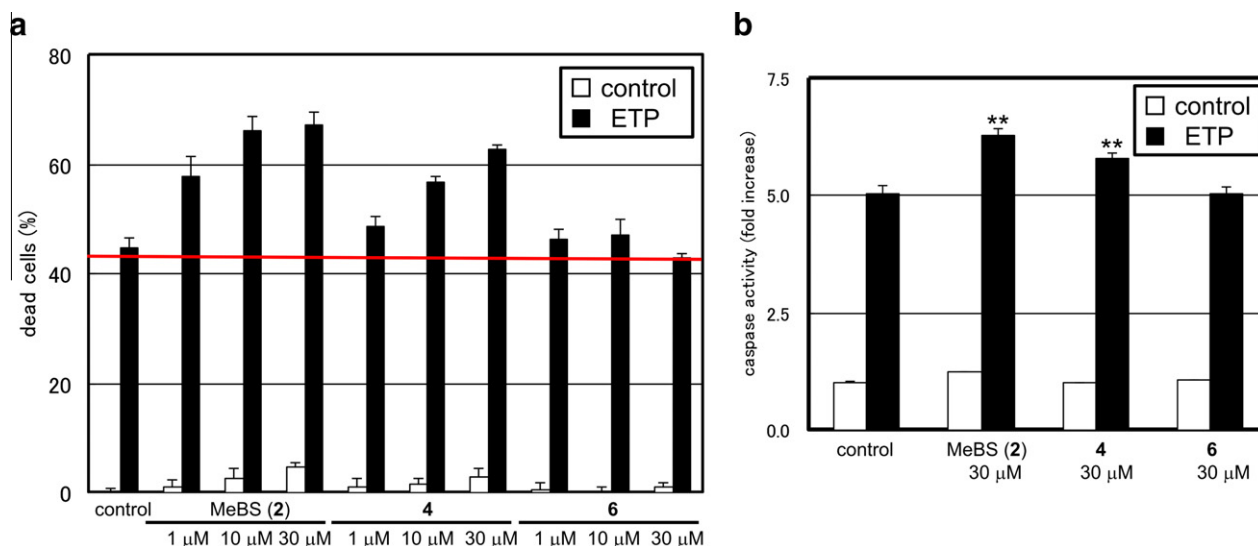
#### 4. Conclusion

In conclusion, the previously reported ester-type SNIPER induces degradation of not only its target protein, but also cIAP1, and therefore in the present work we designed amide-type SNIPER to improve the target protein/cIAP1 degradation selectivity. We synthesized the designed amide-type SNIPER, and confirmed that it does indeed shows improved selectivity. This is an interesting example illustrating that modification of just one atom of a bioactive molecule can cause a major change of activity. We also confirmed that amide-type SNIPER is an effective chemical tool to study the involvement of CRABP-II in MycN-mediated caspase activation in living neuroblastoma cells, finding that: (i) degradation of CRABP-II induces caspase activation and inhibits proliferation of neuroblastoma cells, (ii) degradation of both CRABP-II and cIAP1 is more effective than down-regulation of CRABP-II alone to inhibit the cell proliferation. Amide-type SNIPER should be adaptable to various other target proteins by using appropriate specific ligands, and we are currently examining protein knockdown of other target proteins with amide-type SNIPERs. We believe that highly selective target protein knockdown with SNIPERs will provide new insights into the chemical biology of various proteins and open up new strategies for drug therapy.

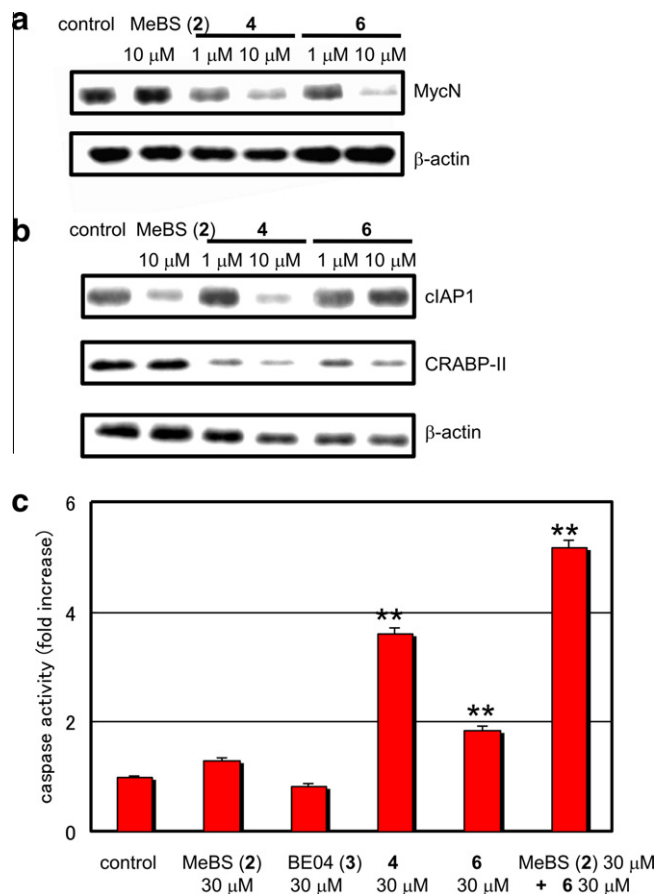
#### 5. Experimental section

##### 5.1. Chemistry

Proton nuclear magnetic resonance spectra ( $^1\text{H}$  NMR) and carbon nuclear magnetic resonance spectra ( $^{13}\text{C}$  NMR) were recorded on a JEOL JNMGX500 (500 MHz) spectrometer in the indicated solvent. Chemical shifts ( $\delta$ ) are reported in parts per million relative to the internal standard tetramethylsilane. High-resolution mass spectra (HRMS) and fast atom bombardment (FAB) mass spectra were recorded on a JEOL JMA-HX110 mass spectrometer. Bestatin (1) and methyl bestatin (MeBS, 2) were provided by Nippon Kaya Co., Ltd (Tokyo, Japan). The other chemical reagents and solvents were purchased from Aldrich, Merck, Tokyo Kasei Kogyo, Wako Pure Chemical Industries, and Kanto Kagaku and used without purification. Flash column chromatography was performed



**Figure 10.** (a) LDH assay. HT1080 cells were treated with compounds in the presence or absence of 30 μg/mL etoposide (ETP) for 24 h. The data are the means of triplicate determinations. (b) Caspase activity assay. HT1080 cells were treated with compounds in the presence or absence of 30 μg/mL etoposide (ETP) for 24 h. The data are the means of triplicate determinations. \*\* $P < 0.01$ ; ANOVA and Bonferroni-type multiple  $t$  test results indicated significant differences between ETP control and the combination of ETP with MeBS (2), and between ETP control and the combination of ETP with 4.



**Figure 11.** (a) Western blotting detection of MycN in IMR-32 cells. Western blot-detection of MycN levels in IMR-32 cells after 48-h treatment with MeBS (2), 4, and 6. (b) Western blot-detection of cIAP1 and CRABP-II levels in IMR-32 cells after 24-h treatment with MeBS (2), SNIPER (4), and SNIPER (6). (c) Caspase activity assay. IMR-32 cells were treated with compounds for 24 h. The data are the means of triplicate determinations. \*\* $P < 0.01$ ; ANOVA and Bonferroni-type multiple  $t$  test results indicated significant differences from the control for 4, 6 and the combination of MeBS (2) with 6.

using Silica Gel 60 (particle size 0.060–0.210 mm) supplied by Kan-to Kagaku.

#### 5.1.1. (S)-Methyl 4-methyl-2-(4-phenylbutanamido)pentanoate (12)

EDCI (2.61 g, 13.6 mmol) was added to a solution of leucine methyl ester hydrochloride (10) (2.45 g, 13.5 mmol), 4-phenylbutanoic acid (11) (1.50 g, 9.14 mmol), HOBT-H<sub>2</sub>O (2.86 g, 18.7 mmol) and Et<sub>3</sub>N (5.60 mL, 40.4 mmol) in THF (150 mL) with cooling in an ice-bath. The resulting mixture was stirred at room temperature for 5 h, then concentrated, and the residue was extracted with AcOEt. The organic solution was washed with 10% aqueous citric acid, saturated NaHCO<sub>3</sub> and brine, and dried over MgSO<sub>4</sub>. Filtration and evaporation of the solvent in vacuo gave 2.65 g (100%) of 12 as a colorless oil; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz,  $\delta$ ; ppm): 7.30–7.16 (5H, m), 5.74 (1H, br d,  $J = 7.3$  Hz), 4.65 (1H, m), 3.73 (3H, s), 2.66 (2H, t,  $J = 7.3$  Hz), 2.21 (2H, t,  $J = 7.3$  Hz), 1.98 (2H, quin,  $J = 7.3$  Hz), 1.54 (3H, m), 0.94 (3H, d,  $J = 5.7$  Hz), 0.93 (3H, d,  $J = 5.7$  Hz); MS (FAB)  $m/z$ : 292 (MH<sup>+</sup>).

#### 5.1.2. Preparation of (S)-2-[(2S,3R)-3-(tert-butoxycarbonyl)-2-hydroxy-4-phenylbutanamido]-4-methylpentanoic acid (N-Boc bestatin, 13)

To a suspension of bestatin (1) (950 mg, 3.08 mmol) in acetone (140 mL) was added 2 N aqueous NaOH (3.10 mL, 6.20 mmol) and

(Boc)<sub>2</sub>O (1.40 g, 6.41 mmol) with cooling in an ice-bath. The resulting mixture was stirred at room temperature for 24 h, then concentrated in vacuo, and the residue was dissolved in AcOEt. The organic solution was washed with 10% aqueous citric and brine, and dried over MgSO<sub>4</sub>. Filtration, evaporation of the solvent in vacuo and purification of the residue by flash column chromatography (AcOEt/*n*-hexane = 1:2 to AcOEt only) gave 770 mg (61%) of 13 as a colorless solid; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 500 MHz,  $\delta$ ; ppm): 7.74 (1H, d,  $J = 8.5$  Hz), 7.27–7.16 (5H, m), 6.12 (1H, d,  $J = 9.3$  Hz), 6.02 (1H, m), 4.28 (1H, m), 3.90 (1H, m), 3.82 (1H, m), 2.78 (1H, dd,  $J = 13.1, 7.0$  Hz), 2.63 (1H, dd,  $J = 13.1, 7.9$  Hz), 1.64–1.59 (2H, m), 1.47 (1H, m), 1.27 (9H, s), 0.86 (3H, d,  $J = 6.0$  Hz), 0.81 (3H, d,  $J = 6.1$  Hz); MS (FAB)  $m/z$ : 431 (MNa<sup>+</sup>), 409 (MH<sup>+</sup>).

#### 5.1.3. (R)-2-Isobutyl-4-oxo-7-phenylheptanoic acid (14)

LiOH-H<sub>2</sub>O (823 mg, 19.6 mmol) was added to a solution of 12 (2.48 g, 8.51 mmol) in MeOH/H<sub>2</sub>O (60.0 mL/30.0 mL) and the resulting mixture was stirred at room temperature for 15 h. Then the reaction mixture was acidified with 2 N HCl and concentrated in vacuo. The residue was extracted with AcOEt. The organic solution was washed with brine, and dried over MgSO<sub>4</sub>. Filtration and evaporation of the solvent in vacuo gave a crude solid, which was suspended in AcOEt. Insoluble material was collected by filtration to give 2.20 g (93%) of 14 as a colorless solid; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 500 MHz,  $\delta$ ; ppm): 12.43 (1H, br), 8.04 (1H, d,  $J = 7.9$  Hz), 7.26 (2H, t,  $J = 7.9$  Hz), 7.16 (3H, m), 4.21 (1H, m), 2.54 (2H, t,  $J = 7.3$  Hz), 2.12 (2H, d,  $J = 7.3$  Hz), 1.75 (2H, quin,  $J = 7.3$  Hz), 1.63 (1H, m), 1.49 (2H, m), 0.87 (3H, d,  $J = 6.7$  Hz), 0.83 (3H, d,  $J = 6.7$  Hz); MS (FAB)  $m/z$ : 278 (MH<sup>+</sup>).

#### 5.1.4. tert-Butyl (2R,3S)-3-hydroxy-4-[(S)-4-methyl-1-(methylamino)-1-oxopentan-2-ylamino]-4-oxo-1-phenylbutan-2-ylcarbamate (15)

EDCI (72.0 mg, 0.376 mmol) was added to a solution of 14 (104 mg, 0.255 mmol), methylamine hydrochloride (75.0 mg, 1.11 mmol), HOBT-H<sub>2</sub>O (78.0 mg, 0.509 mmol) and *i*Pr<sub>3</sub>N (220  $\mu$ L, 1.26 mmol) in DMF/CH<sub>2</sub>Cl<sub>2</sub> (4.00 mL/3.00 mL) with cooling in an ice-bath. The resulting mixture was stirred at room temperature for 22 h, then quenched with water, and extracted with AcOEt. The organic layer was washed with brine, and dried over MgSO<sub>4</sub>. Filtration, evaporation of the solvent in vacuo and purification of the residue by flash column chromatography (AcOEt only) gave 94.0 mg (88%) of 15 as a colorless solid; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz,  $\delta$ ; ppm): 7.29 (2H, t,  $J = 6.7$  Hz), 7.23 (3H, m), 6.97 (1H, d,  $J = 8.5$  Hz), 6.36 (1H, br), 5.44 (1H, d,  $J = 4.9$  Hz), 4.98 (1H, d,  $J = 7.9$  Hz), 4.44 (1H, m), 4.11 (2H, dd,  $J = 7.3, 3.0$  Hz), 4.00 (1H, m), 3.11 (1H, m), 3.04 (1H, m), 2.78 (1.5H, s), 2.77 (1.5H, s), 1.65 (3H, m), 1.39 (9H, s), 0.93 (3H, d,  $J = 6.0$  Hz), 0.89 (3H, d,  $J = 6.1$  Hz); MS (FAB)  $m/z$ : 444 (MNa<sup>+</sup>), 422 (MH), 322 (MH<sup>+</sup>–Boc).

#### 5.1.5. (S)-2-[(2S,3R)-3-Amino-2-hydroxy-4-phenylbutanamido]-N,4-dimethylpentanamide hydrochloride (BE04, 5)

4 N HCl in 1,4-dioxane (500  $\mu$ L, 2.00 mmol) was added to 15 (63.2 mg, 0.150 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (1.00 mL) with cooling in an ice-bath and the resulting mixture was stirred at room temperature for 2 h, then concentrated in vacuo to give 53.2 mg (99%) of 5 as a colorless solid; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 500 MHz,  $\delta$ ; ppm): 8.04–7.97 (5H, m), 7.34–7.23 (5H, m), 6.66 (1H, d,  $J = 6.1$  Hz), 4.22 (1H, m), 3.99 (1H, m), 3.53 (1H, br), 2.95–2.82 (2H, m), 2.54 (1.5H, s), 2.53 (1.5H, s), 1.52–1.42 (3H, m), 0.87 (3H, d,  $J = 6.1$  Hz), 0.84 (3H, d,  $J = 6.7$  Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 125 MHz,  $\delta$ ; ppm): 171.97, 170.64, 136.27, 129.44, 128.65, 126.94, 68.18, 54.20, 51.24, 40.92, 34.70, 25.55, 24.24, 22.85, 21.91; MS (FAB)  $m/z$ : 322 (MH<sup>+</sup>); HRMS (FAB) calcd for C<sub>17</sub>H<sub>28</sub>N<sub>3</sub>O<sub>3</sub><sup>+</sup>, 322.2131; found 322.2115.



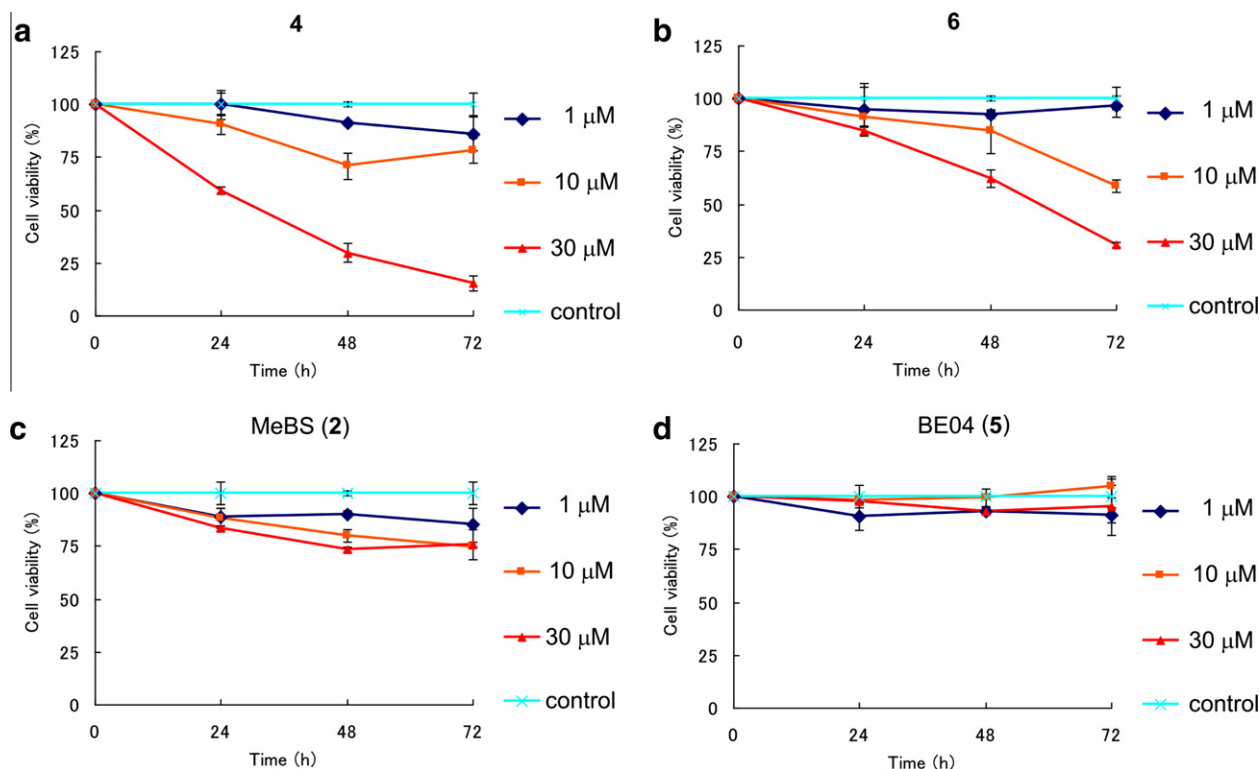


Figure 12. Viability of IMR-32 cells treated with (a) **4**, (b) **6**, (c) MeBS (**2**), and (d) BE04 (**5**).

#### 5.1.6. (S)-N,4-Dimethyl-2-(4-phenylbutanamido)pentanamide (**7**)

Compound **7** (yield; 106 mg, 99%) was prepared from **14** (102 mg, 0.368 mmol), methylamine hydrochloride (134 mg, 1.98 mmol) using the procedure described for **15**; yellow solid;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz,  $\delta$ ; ppm): 7.28 (2H, t,  $J = 7.3$  Hz), 7.21 (1H, d,  $J = 7.3$  Hz), 7.17 (2H, d,  $J = 7.3$  Hz), 6.10 (1H, br), 5.83 (1H, d,  $J = 8.5$  Hz), 4.41 (1H, m), 2.80 (1.5H, s), 2.79 (1.5H, s), 2.63 (2H, t,  $J = 7.6$  Hz), 2.20 (2H, t,  $J = 7.3$  Hz), 1.95 (2H, quin,  $J = 7.6$  Hz), 1.66–1.49 (3H, m), 0.93 (3H, d,  $J = 5.4$  Hz), 0.92 (3H, d,  $J = 6.0$  Hz);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 125 MHz,  $\delta$ ; ppm): 173.02, 172.75, 141.49, 128.68, 128.63, 126.29, 51.64, 41.24, 35.90, 35.32, 27.19, 26.49, 24.96, 23.04, 22.43; MS (FAB)  $m/z$ : 291 ( $\text{MH}^+$ ); HRMS (FAB) calcd for  $\text{C}_{17}\text{H}_{27}\text{N}_2\text{O}_2^+$ , 291.2073; found 291.2076.

#### 5.1.7. 5-(Dimethylamino)-N-(2-hydroxyethyl)naphthalene-1-sulfonamide (**19**)

2-Aminoethanol (**18**) (250  $\mu\text{L}$ , 4.14 mmol) was added to a solution of 5-(dimethylamino)naphthalene-1-sulfonyl chloride (**17**) (216 mg, 0.801 mmol) and  $\text{Et}_3\text{N}$  (600  $\mu\text{L}$ , 4.33 mmol) in  $\text{CH}_2\text{Cl}_2$  (20.0 mL) with cooling by an ice-bath and the resulting mixture was stirred at room temperature for 2 h, then poured into water. The organic layer was washed with brine and dried over  $\text{MgSO}_4$ . Filtration, evaporation of the solvent in vacuo and purification of the residue by flash column chromatography ( $\text{CH}_2\text{Cl}_2/\text{MeOH} = 10:1$ ) gave 160 mg (68%) of **19** as a fluorescent powder;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz,  $\delta$ ; ppm): 8.55 (1H, d,  $J = 8.5$  Hz), 8.28 (1H, d,  $J = 8.5$  Hz), 8.26 (1H, d,  $J = 7.3$  Hz), 7.58 (1H, t,  $J = 8.2$  Hz), 7.53 (1H, t,  $J = 7.3$  Hz), 7.20 (1H, d,  $J = 7.3$  Hz), 5.20 (1H, br d,  $J = 7.3$  Hz), 3.60 (2H, q,  $J = 4.8$  Hz), 3.04 (2H, q,  $J = 5.9$  Hz), 2.89 (6H, s), 1.89 (1H, br); MS (FAB)  $m/z$ : 294 ( $\text{M}^+$ ).

#### 5.1.8. (S)-2-[1-(Dimethylamino)naphthalene-5-sulfonamido]ethyl 2-[(2S,3R)-3-(tert-butoxycarbonyl)-2-hydroxy-4-phenylbutanamido]-4-methylpentanoate (**20**)

Compound **20** (yield; 83.2 mg, 24%) was prepared from *N*-Boc bestatin (**13**) (202 mg, 0.495 mmol) and alcohol **19** (108 mg,

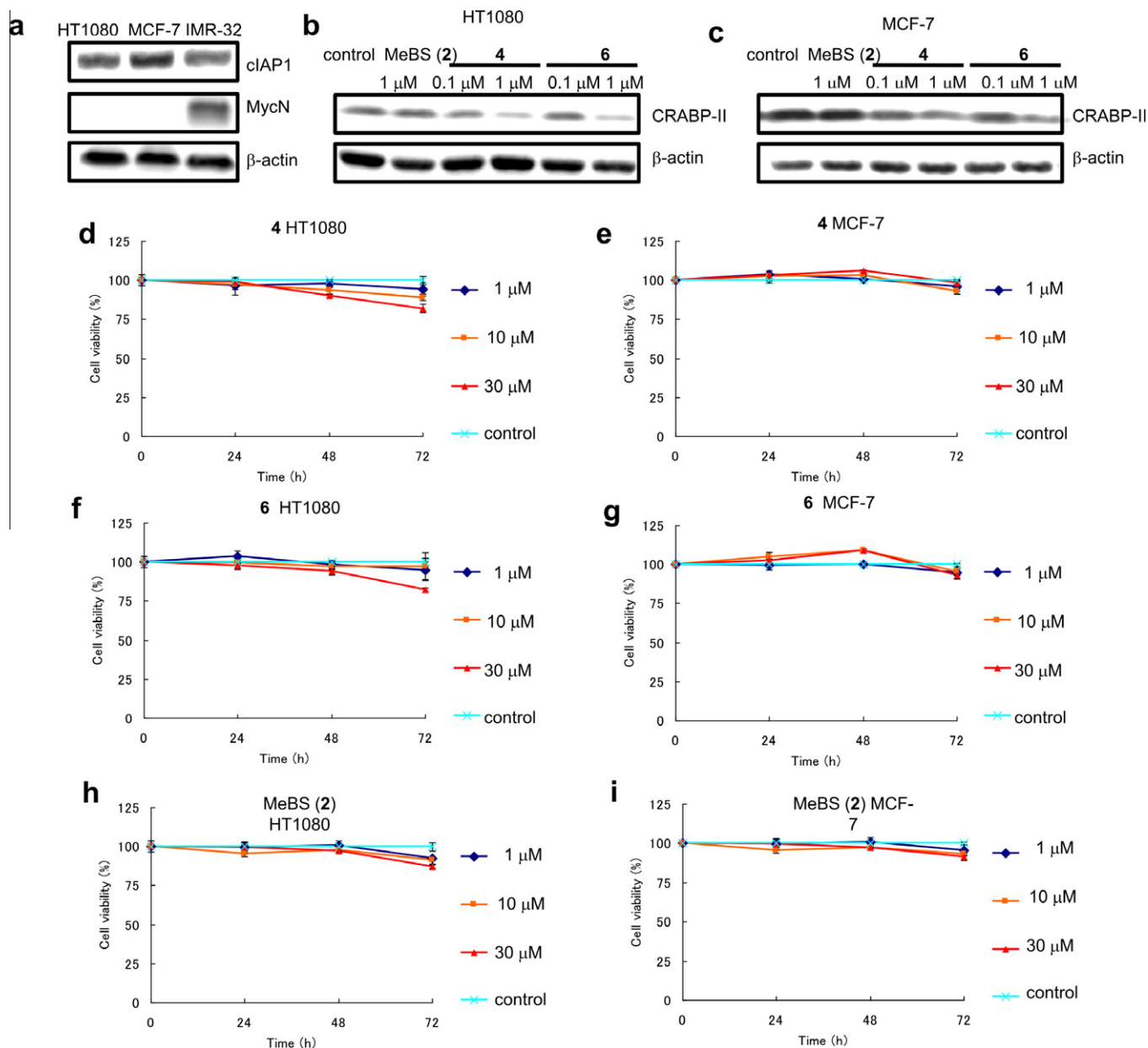
0.367 mmol) using the procedure described for **15**; fluorescent solid;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz,  $\delta$ ; ppm): 8.55 (1H, d,  $J = 8.5$  Hz), 8.28 (1H, d,  $J = 8.5$  Hz), 8.22 (1H, dd,  $J = 7.0$ , 1.2 Hz), 7.53 (1H, t,  $J = 7.8$  Hz), 7.50 (1H, t,  $J = 7.3$  Hz), 7.35–7.15 (6H, m), 5.83 (1H, m), 5.45 (1H, m), 5.17 (1H, br d,  $J = 6.7$  Hz), 4.40 (1H, m), 4.20–4.12 (2H, m), 4.11–4.05 (2H, m), 3.25–3.00 (4H, m), 2.89 (6H, s), 1.63–1.52 (3H, m), 1.37 (9H, s), 0.91 (3H, d,  $J = 6.1$  Hz), 0.89 (3H, d,  $J = 6.1$  Hz); MS (FAB)  $m/z$ : 684 ( $\text{M}^+$ ), 585 ( $\text{MH}^+ - \text{Boc}$ ).

#### 5.1.9. (S)-2-[1-(Dimethylamino)naphthalene-5-sulfonamido]ethyl 2-[(2S,3R)-3-amino-2-hydroxy-4-phenylbutanamido]-4-methylpentanoate dihydrochloride (DanBE-2HCl, **16-2HCl**)

Compound **9-2HCl** (yield; 42.6 mg, 100%) was prepared from **20** (44.6 mg, 0.0651 mmol) using the procedure described for **5**; fluorescent powder;  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ , 500 MHz,  $\delta$ ; ppm): 8.66 (1H, br d,  $J = 8.5$  Hz), 8.43 (1H, br d,  $J = 7.9$  Hz), 8.31 (2H, m), 8.13 (1H, d,  $J = 6.7$  Hz), 8.04 (3H, br), 7.68 (1H, t,  $J = 7.9$  Hz), 7.66 (1H, t,  $J = 7.3$  Hz), 7.54 (1H, br), 7.30–7.20 (5H, m), 4.22 (1H, m), 4.00 (1H, d,  $J = 3.6$  Hz), 3.93 (2H, m), 3.49 (1H, m), 3.06–2.82 (10H, m), 1.65–1.40 (3H, m), 0.86 (3H, d,  $J = 6.1$  Hz), 0.82 (3H, d,  $J = 6.1$  Hz);  $^{13}\text{C}$  NMR ( $\text{DMSO}-d_6$ , 125 MHz,  $\delta$ ; ppm): 171.89, 171.12, 136.36, 136.21, 129.42, 128.88, 128.84, 128.58, 128.32, 128.09, 127.78, 126.86, 124.39, 68.26, 66.34, 63.34, 54.27, 50.39, 45.49, 41.12, 34.50, 24.16, 22.65, 21.54; MS (FAB)  $m/z$ : 585 ( $\text{MH}^+$ ); HRMS (FAB) calcd for  $\text{C}_{30}\text{H}_{41}\text{N}_4\text{O}_6\text{S}^+$ , 585.2747; found 585.2727.

#### 5.1.10. tert-Butyl 2-[2-(2-azidoethoxy)ethoxy]ethylcarbamate (**22**)

A mixture of *p*-toluenesulfonyl chloride (583 mg, 3.06 mmol) in  $\text{CH}_2\text{Cl}_2$  (20.0 mL) was added to a solution of **21** (**380** mg, 1.52 mmol),  $\text{Et}_3\text{N}$  (500  $\mu\text{L}$ , 3.61 mmol) and DMAP (37.0 mg, 0.303 mmol)  $\text{CH}_2\text{Cl}_2$  (2.00 mL) in a dropwise fashion over a period of 1 h. The resulting mixture was stirred at room temperature for 1 h, then poured into water. The organic layer was washed with brine, and dried over  $\text{MgSO}_4$ . Filtration, evaporation of the solvent



**Figure 13.** (a) Western blot-detection of cIAP1 and MycN levels in HT1080 cells, MCF-7 cells and IMR-32 cells. (b) Western blot-detection of CRABP-II levels in HT1080 cells after 6-h treatment with MeBS (2), ester-type SNIPER (4) and amide-type SNIPER (6). (c) Western blot-detection of CRABP-II levels in MCF-7 cells after 24-h treatment with MeBS (2), ester-type SNIPER (4), and amide-type SNIPER (6). (d–i) Viability of HT1080 and MCF-7 cells treated with MeBS (2), ester-type SNIPER (4) and amide-type SNIPER (6).

in vacuo and purification of the residue by flash column chromatography (AcOEt/*n*-hexane = 1:1 AcOEt only) gave 515 mg (84%) of **22** as a colorless oil. A suspension of **22** (500 mg, 1.24 mmol) and  $\text{NaN}_3$  (180 mg, 2.77 mmol) in DMF (5.00 mL) was stirred at 60 °C for 8 h. Then, the reaction mixture was poured into water and extracted with AcOEt. The organic layer was washed with brine, and dried over  $\text{MgSO}_4$ . Filtration and evaporation of the solvent in vacuo gave 335 mg (98%) of **23** as a colorless oil;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz,  $\delta$ ; ppm): 5.01 (1H, br), 3.69 (6H, m), 3.56 (2H, t,  $J$  = 4.9 Hz), 3.41 (2H, t,  $J$  = 7.5 Hz), 3.32 (2H, m); 1.44 (9H, s); MS (FAB)  $m/z$ : 297 ( $\text{MNa}^+$ ), 275 ( $\text{MH}^+$ ), 175 ( $\text{MH}^+ - \text{Boc}$ ).

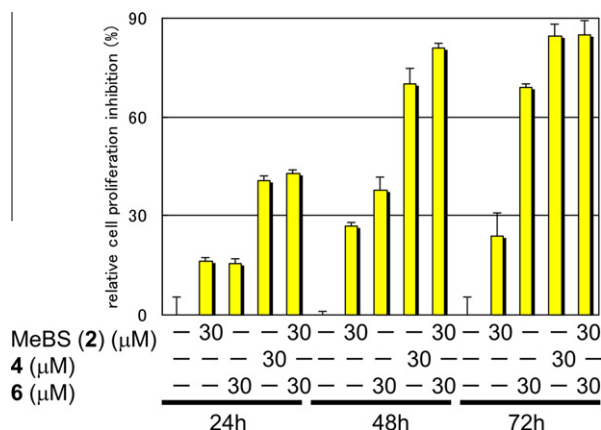
#### 5.1.11. *tert*-Butyl 2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate (**24**)

10% Pd/C (20.2 mg) was added to a solution of **23** (211 mg, 0.769 mmol) in EtOH (5.00 mL). After having been stirred at room

temperature under  $\text{H}_2$  for 13 h, the reaction mixture was filtered though Celite. The solvent was removed by concentration in vacuo to give crude **24** (191 mg) as a colorless oil, which was used in the next reaction without further purification; MS (FAB)  $m/z$ : 249 ( $\text{MH}^+$ ).

#### 5.1.12. (S)-2-[2-[2-(*tert*-Butoxycarbonyl)ethoxy]ethoxy]ethyl 2-((2S,3R)-3-[[[9H-fluoren-9-yl)methoxy]carbonyl]-2-hydroxy-4-phenyl-butanamido)-4-methylpentanamide (**26**)

Compound **26** (56.0 mg, 78%; from **25**) was prepared from crude amine (**24**) (140 mg) and **25**<sup>2</sup> (50.1 mg, 0.0944 mmol) using the procedure described for **15**; colorless oil;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz,  $\delta$ ; ppm): 7.76 (2H, d,  $J$  = 7.3 Hz), 7.51 (2H, m), 7.38 (2H, t,  $J$  = 7.6 Hz), 7.31–7.14 (7H, m), 6.70 (1H, br), 5.80 (1H, br d,  $J$  = 6.1 Hz), 5.48 (1H, m), 5.43 (1H, m), 5.12 (1H, m), 4.48 (1H, m), 4.43–4.20 (5H, m), 3.54–3.40 (10H, m), 3.28 (2H, br q,  $J$  = 4.3 Hz),



**Figure 14.** Relative inhibition of proliferation of IMR-32 cells by treatment with MeBS (**2**), SNIPER (**4**), SNIPER (**6**), and the combination of MeBS (**2**) and SNIPER (**6**). The graph was drawn based on calculated IMR-32 cells viability. SNIPER (**4**) or the combination of SNIPER (**6**) and MeBS (**2**) inhibited proliferation of IMR-32 cells more strongly than did SNIPER (**6**). The activity of the combination of SNIPER (**6**) and MeBS (**2**) was similar to that of SNIPER (**4**).

3.05 (2H, m), 1.62 (3H, m), 1.44 (9H, s), 0.93 (3H, d,  $J = 6.0$  Hz), 0.89 (3H, d,  $J = 6.1$  Hz); MS (FAB)  $m/z$ : 761 ( $MH^+$ ), 661 ( $MH^+ - Boc$ ).

#### 5.1.13. (S)-tert-Butyl 2-(2-{2-[4-methyl-2-(4-phenylbutanamido)pentanamido]ethoxy}ethoxy)ethyl-carbamate (**27**)

Compound **27** (yield; 47.5 mg, 88%) was prepared from **14** (29.5 mg, 0.106 mmol) and crude amine (**24**) (50.0 mg) using the procedure described for **15**; colorless oil;  $^1H$  NMR ( $CDCl_3$ , 500 MHz,  $\delta$ ; ppm): 7.27 (2H, t,  $J = 7.3$  Hz), 7.18 (1H, t,  $J = 7.3$  Hz), 7.17 (2H, d,  $J = 6.7$  Hz), 6.65 (1H, m), 6.02 (1H, m), 5.20 (1H, m), 4.47 (1H, m), 3.76–3.44 (10H, m), 3.32 (2H, m), 2.63 (2H, t,  $J = 7.6$  Hz), 2.21 (2H, t,  $J = 7.3$  Hz), 1.95 (2H, quin,  $J = 7.6$  Hz), 1.62 (2H, m), 1.59 (1H, m), 1.43 (9H, s), 0.93 (3H, d,  $J = 6.1$  Hz); MS (FAB)  $m/z$ : 508 ( $MH^+$ ), 408 ( $MH^+ - Boc$ ).

#### 5.1.14. (9H-Fluoren-9-yl)methyl (2R,3S)-4-((S)-1-{2-[2-(2-aminoethoxy)ethoxy]ethylamino}-4-methyl-1-oxopentan-2-ylamino)-3-hydroxy-4-oxo-1-phenylbutan-2-ylcarbamate hydrochloride (**29-HCl**)

4 N HCl in 1,4-dioxane (250  $\mu$ L, 1 mmol) was added to **26** (56.1 mg, 0.0736 mmol) with cooling in an ice-bath and the resulting mixture was stirred at room temperature for 1 h. The reaction mixture was concentrated in vacuo to give crude **29-HCl** (51.2 mg), which was used in the next step without further purification; MS (FAB)  $m/z$ : 661 ( $MH^+ - HCl$ ).

#### 5.1.15. (S)-N-{2-[2-(2-Aminoethoxy)ethoxy]ethyl}-4-methyl-2-(4-phenylbutanamido)pentanamide hydrochloride (**30-HCl**)

Crude **30-HCl** was prepared from **27** (47.5 mg, 0.0936 mmol) using the procedure described for **29-HCl**. Crude **30-HCl** (45.3 mg) was used in next step without further purification.

#### 5.1.16. (2E,4E,6E,8E)-2-Cyanoethyl 9-((E)-3-{2-[2-(2-[(S)-2-((2S,3R)-3-[(9H-fluoren-9-yl)methoxy]carbonyl)-2-hydroxy-4-phenylbutanamido]-4-methylpentanamido]ethoxy}ethoxy)ethylamino]-2-oxoethoxyimino)-2,6,6-trimethylcyclohex-1-enyl)-3,7-dimethylnona-2,4,6,8-tetraenoate (**31**)

Compound **31** (40.3 mg, 56% from **28**) was prepared from **28**<sup>2</sup> (29.2 mg, 0.0662 mmol) and crude **29-HCl** (51.2 mg) using the procedure described for **12**; yellow oil;  $^1H$  NMR ( $CDCl_3$ , 500 MHz,  $\delta$ ; ppm): 7.72 (2H, d,  $J = 7.3$  Hz), 7.46 (2H, d,  $J = 7.3$  Hz), 7.35 (2H, t,

$J = 7.3$  Hz), 7.34–7.22 (7H, m), 7.00 (1H, dd,  $J = 15.2$ , 11.0 Hz), 6.80 (1H, br), 6.66 (1H, m), 6.32–6.16 (4H, m), 5.78 (1H, s), 5.48 (1H, d,  $J = 8.5$  Hz), 5.36 (1H, d,  $J = 5.5$  Hz), 4.55 (2H, s), 4.36 (1H, m), 4.34 (7H, m), 3.60 (10H, m), 3.37 (2H, m), 2.98 (2H, m), 2.70 (2H, t,  $J = 6.1$  Hz), 2.63 (2H, t,  $J = 6.4$  Hz), 2.33 (3H, s), 2.01 (3H, s), 1.83 (3H, s), 1.67 (1H, m), 1.58 (4H, m), 1.06 (6H, s), 0.84 (3H, d,  $J = 6.0$  Hz), 0.81 (3H, d,  $J = 6.1$  Hz); MS (FAB)  $m/z$ : 1083 ( $M^+$ ).

#### 5.1.17. (2E,4E,6E,8E)-2-Cyanoethyl 3,7-dimethyl-9-((E)-2,6,6-trimethyl-3-{2-[2-(2-[(S)-4-methyl-2-(4-phenylbutanamido)pentanamido]ethoxy}ethoxy)ethylamino]-2-oxoethoxyimino]cyclohex-1-enyl)nona-2,4,6,8-tetraenoate (**32**)

Compound **32** (yield; 23.2 mg, 62% from **28**) was prepared from **28** (20.0 mg, 0.454 mmol) and crude **30-HCl** (45.3 mg) using the procedure described for **12**; yellow solid;  $^1H$  NMR ( $CDCl_3$ , 500 MHz,  $\delta$ ; ppm): 7.27 (2H, t,  $J = 7.3$  Hz), 7.19 (1H, t,  $J = 7.3$  Hz), 7.16 (2H, d,  $J = 7.3$  Hz), 7.03 (1H, dd,  $J = 14.6$ , 11.6 Hz), 6.68 (1H, br), 6.57 (1H, br), 6.36–6.20 (4H, m), 5.82 (1H, s), 4.58 (2H, s), 4.45 (1H, m), 4.33 (2H, t,  $J = 6.1$  Hz), 3.59–3.40 (12H, m), 2.73 (2H, t,  $J = 6.1$  Hz), 2.66 (4H, m), 2.37 (3H, s), 2.21 (2H, t,  $J = 7.3$  Hz), 2.03 (3H, s), 1.95 (2H, quin,  $J = 7.9$  Hz), 1.86 (3H, s), 1.68–1.54 (5H, m), 1.09 (6H, s), 0.93 (3H, d,  $J = 2.4$  Hz), 0.92 (3H, d,  $J = 2.4$  Hz); MS (FAB)  $m/z$ : 830 ( $MH^+$ ).

#### 5.1.18. (2E,4E,6E,8E)-9-[(E)-3-(2-[2-(2-[(S)-2-[(2S,3R)-3-Amino-2-hydroxy-4-phenylbutanamido]-4-methylpentanamido]ethoxy)ethoxy]ethylamino)-2-oxoethoxyimino]-2,6,6-trimethylcyclohex-1-enyl]-3,7-dimethylnona-2,4,6,8-tetraenoic acid (amide-type SNIPER, **6**)<sup>2-4</sup>

1 N TBAF in THF (370  $\mu$ L, 0.370 mmol) was added to a solution of **31** (40.3 mg, 0.0372 mmol) and MeOH (30.0  $\mu$ L, 0.741 mmol). After having been stirred at room temperature for 1 h, the mixture was purified by flash column chromatography ( $CHCl_3$ /MeOH = 20:1) to give 33 mg of crude acid as a yellow oil. DBU (17.0  $\mu$ L, 0.114 mmol) was added to a solution of the crude acid and dodecyl mercaptan (13.0  $\mu$ L, 0.0552 mmol) in  $CH_2Cl_2$  (1.00 mL). After having been stirred at room temperature for 1 h, the mixture was purified by flash column chromatography ( $CHCl_3$ /MeOH = 9:1) and PTLC ( $CHCl_3$ /MeOH/ $NH_3$  aqueous = 3:1:0.1) to give 17.2 mg (57%; two steps) of **6** as a yellow oil;  $^1H$  NMR ( $CDCl_3$ , 500 MHz,  $\delta$ ; ppm): 7.87 (1H, d,  $J = 6.7$  Hz), 7.30–7.24 (5H, m), 7.01 (1H, m), 6.97 (1H, dd,  $J = 15.2$ , 12.1 Hz), 6.73 (1H, s), 6.38–6.18 (4H, m), 5.82 (1H, s), 4.58 (2H, s), 4.44 (1H, m), 4.07 (1H, m), 3.72–3.50 (13H, m), 3.33 (2H, m), 3.02 (2H, m), 2.66 (2H, t,  $J = 6.7$  Hz), 2.33 (3H, s), 2.02 (3H, s), 1.86 (3H, s), 1.67 (5H, m), 1.09 (6H, s), 0.93 (3H, d,  $J = 6.1$  Hz), 0.92 (3H, d,  $J = 6.0$  Hz);  $^{13}C$  NMR ( $CDCl_3$ , 125 MHz,  $\delta$ ; ppm): 173.34, 172.02, 170.67, 158.88, 150.34, 139.35, 138.38, 138.06, 136.72, 131.64, 129.34, 128.75, 126.74, 126.61, 124.79, 73.03, 70.32, 70.21, 69.92, 69.76, 54.64, 51.84, 40.63, 39.31, 38.80, 35.97, 34.88, 27.62, 24.88, 23.02, 21.73, 20.23, 14.91, 13.86, 12.82; MS (FAB)  $m/z$ : 808 ( $MH^+$ ); HRMS (FAB) calcd for  $C_{44}H_{66}N_5O_9^+$ , 808.4861; found 808.4825.

#### 5.1.19. (2E,4E,6E,8E)-3,7-Dimethyl-9-((E)-2,6,6-trimethyl-3-{2-[2-(2-[(S)-4-methyl-2-(4-phenylbutanamido)pentanamido]ethoxy}ethoxy)ethylamino]-2-oxoethoxyimino]cyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid (**8**)

1 N TBAF in THF (280  $\mu$ L, 0.280 mmol) was added to a solution of **32** (23.2 mg, 0.0279 mmol) and MeOH (22.0  $\mu$ L, 0.543 mmol) in THF (560  $\mu$ L). The mixture was stirred at room temperature for 2 h, then purified by flash column chromatography ( $CHCl_3$  only to  $CHCl_3$ /MeOH = 20:1) and PTLC ( $CHCl_3$ /MeOH = 20:1) to give 15.1 mg (70%) of **8** as a yellow oil;  $^1H$  NMR ( $CDCl_3$ , 500 MHz,  $\delta$ ;

ppm): 7.25 (2H, t,  $J = 7.3$  Hz), 7.17 (1H, t,  $J = 7.3$  Hz), 7.14 (2H, d,  $J = 6.7$  Hz), 7.01 (1H, dd,  $J = 15.2, 11.6$  Hz), 6.68 (1H, br), 6.61 (1H, br), 6.35–6.17 (4H, m), 5.82 (1H, s), 4.56 (2H, s), 4.45 (1H, m), 3.89–3.32 (12H, m), 2.65–2.58 (4H, m), 2.34 (3H, s), 2.20 (2H, t,  $J = 6.7$  Hz), 2.00 (3H, s), 1.93 (2H, quin,  $J = 7.3$  Hz), 1.84 (3H, s), 1.59–1.47 (5H, m), 1.07 (6H, s), 0.93 (3H, d,  $J = 6.1$  Hz), 0.92 (3H, d,  $J = 6.1$  Hz);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 125 MHz,  $\delta$ ; ppm): 172.84, 172.43, 170.47, 169.97, 158.76, 154.31, 150.15, 141.40, 139.21, 138.90, 136.25, 131.44, 131.06, 128.47, 128.39, 126.89, 125.98, 124.92, 118.32, 73.08, 70.40, 70.21, 69.91, 69.69, 51.55, 41.51, 39.29, 38.76, 35.99, 35.18, 34.87, 27.62, 27.11, 24.80, 22.88, 22.15, 20.22, 14.87, 13.95, 12.85; MS (FAB)  $m/z$ : 777 ( $\text{MH}^+$ ); HRMS (FAB) calcd for  $\text{C}_{44}\text{H}_{65}\text{N}_4\text{O}_8^+$ , 777.4802; found 777.4843.

## 5.2. Biology

### 5.2.1. Cell culture conditions

Human fibrosarcoma HT1080 cells were cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), penicillin, and streptomycin mixture at 37 °C in a humidified atmosphere of 5%  $\text{CO}_2$  in air. Human neuroblastoma IMR-32 cells were cultured in Eagle's minimal essential medium with non-essential amino acids and 10% FBS at 37 °C in a humidified atmosphere of 5%  $\text{CO}_2$  in air. Human embryonic kidney (HEK) 293 cells and human mammary tumor MCF-7 cells were cultured in D-MEM medium containing 5% FBS and 10% FBS, respectively, at 37 °C in a humidified atmosphere of 5%  $\text{CO}_2$  in air.

### 5.2.2. FLAG-IAPs transfection

The transfection experiments were carried out according to the method reported in Ref. 6.

### 5.2.3. Western blotting

HT1080, FLAG-IAPs HT1080, IMR-32 and MCF-7 ( $1 \times 10^6$ ) cells were treated for the indicated period with MeBS (**2**) (Nippon Kaya Co., Ltd), ATRA (**3**) (Tokyo Kasei Kogyo), MG132 (Peptide Institute Inc.), lactacystin (Toronto Research Chemicals Inc.) and/or synthetic compounds at the indicated concentrations in an appropriate cell culture medium supplemented with 10% FBS, then the cells were collected and extracted with SDS buffer. Protein concentrations of the lysates were determined using a BCA protein assay. Equivalent amounts of protein from each lysate were resolved in 10–20% SDS–polyacrylamide gels and transferred onto PVDF membranes. After blocking with TBS containing 5% skim milk, the transblotted membranes were probed with rabbit polyclonal CRABP-II antibody (Novus Biologicals, Inc.) (1:1000 dilution), affinity-purified goat cIAP1 antibody (R&D systems) (0.5  $\mu\text{g}/\text{mL}$ ), mouse monoclonal RAR $\alpha$  antibody (Perseus Proteomics Inc.) (1:1000 dilution), rabbit MycN antibody (Cell Signal Technology) (1:1000 dilution), anti-goat IgG-horseradish peroxidase conjugates (Kirkegaard & Perry Laboratories, Inc.) (1:5000 dilution), anti-mouse IgG-horseradish peroxidase conjugates (Chemicon) (1:2000 dilution), goat anti-rabbit IgG-horseradish peroxidase conjugates (Amersham) (1:2000 dilution), FLAG antibody (Sigma) (1:500 dilution),  $\beta$ -actin antibody (Santa Cruz Biotechnology, Inc.) (1:2000 dilution) in can-get-signal solution (Toyobo). After probing, the membrane was washed twice more with TBS-T. The immunoblots were visualized by enhanced chemiluminescence with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore).

### 5.2.4. Binding assay

Recombinant GST-BIR3 and GST were produced and purified as described previously.<sup>10</sup> Serial dilutions of GST-BIR3 or GST were incubated in the presence of DanBE (1  $\mu\text{M}$ ) at room temperature for 5 min in phosphate-buffered saline (PBS, pH 7.4). After incubation, the fluorescence polarization of DanBE was measured on

fluorescence spectrometer (Jasco FP-6500; excitation at 335 nm, emission at 550 nm). In competitive displacement studies, MeBS (**2**), BE04 (**5**) or compound **7** was incubated with GST-BIR3 for 5 min before the addition of DanBE.

### 5.2.5. Pull down assay

Pull down assays were carried out according to the method reported in Ref. 3.

### 5.2.6. Reporter gene assay

Reporter gene assays were carried out according to the method reported in Ref. 3.

### 5.2.7. Cell death assay

Etoposide was purchased from Sigma–Aldrich Corporation. CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) was used for cell death assay. HT1080 cells were plated in 96-well plates at the initial density of  $2 \times 10^4$  cells/well (50  $\mu\text{L}/\text{well}$ ) and incubated at 37 °C. After 24 h, cells were exposed to test compounds by adding solutions (50  $\mu\text{L}/\text{well}$ ) of the compounds at various concentrations in medium at 37 °C under 5%  $\text{CO}_2$  in air for 24 h. Lysis buffer (15  $\mu\text{L}$ ) was added to high control wells and the plates were incubated at 37 °C for 45 min. Then 50  $\mu\text{L}$  aliquots were taken from all wells using a multichannel pipette and transferred to fresh 96-well flat-bottomed (enzymatic assay) plates, and 50  $\mu\text{L}$  of reconstituted Substrate Mix was added to each well of the enzymatic assay plate containing samples transferred from the cytotoxicity assay plate. The plates were incubated for 30 min at room temperature, then 50  $\mu\text{L}$  of stop solution was added to each well and the absorbance at 490 nm in each well was measured with an ARVO™ SX microplate reader. The percentage cell growth was calculated from the absorbance readings.

### 5.2.8. Caspase assay

Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega) was used for caspase assay. HT1080 and IMR-32 cells were plated in 96-well plates at the initial density of  $2 \times 10^4$  cells/well (50  $\mu\text{L}/\text{well}$ ) and incubated at 37 °C. After 24 h, cells were exposed to test compounds by adding solutions (50  $\mu\text{L}/\text{well}$ ) of the compounds at various concentrations in medium at 37 °C under 5%  $\text{CO}_2$  in air for 24 h. Then 100  $\mu\text{L}$  of caspase activity detection reagent was added to each well. The plates were further incubated for 10 h (HT1080) or 0.5 h (IMR-32) at room temperature, and the fluorescence in each well was measured with an ARVO™ SX microplate reader (excitation at 485 nm, emission at 535 nm). The fold change of caspase activity was calculated from the fluorescence readings.

### 5.2.9. Cell proliferation inhibition assay

IMR-32, HT1080, and MCF-7 cells were plated in 96-well plates at the initial density of  $2 \times 10^4$  cells/well (50  $\mu\text{L}/\text{well}$ ) and incubated at 37 °C. After 24 h, cells were exposed to test compounds by adding solutions (50  $\mu\text{L}/\text{well}$ ) of the compounds at various concentrations in medium at 37 °C under 5%  $\text{CO}_2$  in air for 0–72 h. The mixtures were then treated with 10  $\mu\text{L}$  of AlamarBlue® (Invitrogen), and incubation was continued at 37 °C for 3 h. The fluorescence in each well was measured with an ARVO™ SX microplate reader (excitation at 540 nm, emission, at 590 nm). The percentage cell growth was calculated from the fluorescence readings.

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### Supplementary data

Supplementary data ( $^1\text{H}$  NMR data) associated with this article can be found, in the online version, at [doi:10.1016/j.bmc.2011.03.057](https://doi.org/10.1016/j.bmc.2011.03.057).

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