SNIPER(TACC3) induces cytoplasmic vacuolization and sensitzes cancer cells to Bortezomib

Nobumichi Ohoka,1 Katsunori Nagai,2 Norihiro Shibata,1 Takayuki Hattori,1 Hiroshi Nara,2 Nobuo Cho2 and Mikihiko Naito1

Key words
Bortezomib, cytoplasmic vacuolization, paraptosis, SNIPER(TACC3), XIAP

Correspondence
Mikihiko Naito, Division of Molecular Target and Gene Therapy Products, National Institute of Health Sciences, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel: +81-3-3700-9428; Fax: +81-3-3707-6950; E-mail: miki-naito@nihs.go.jp

Funding Information
Japan Society for the Promotion of Science (KAKENHI Grants 26680009 to N.O.; 16H05090 and 16K15121 to M.N.), Japan Agency for Medical Research and Development (AMED) (15ak0101029 h1402 and 16ak0101029j1403 to M.N.; 16cm0106124j0001 to N.O.), Ministry of Health and Labour Welfare (to M.N) and Takeda Science Foundation (to N.O.).

Received December 25, 2016; Revised February 6, 2017; Accepted February 7, 2017

Cancer Sci 108 (2017) 1032–1041
doi: 10.1111/cas.13198

Protein knockdown is a recently developed technology to degrade target proteins by a class of chemical compounds such as SNIPERs (Specific and Nongenetic IAP-dependent Protein ERasers) and PROTACs (Proteolysis Targeting Chimeras).1–4) These compounds are chimeric molecules containing a ligand for a target protein of interest connected by a linker to another ligand for a ubiquitin ligase, which are designed to crosslink these proteins to induce polyubiquitylation and proteasomal degradation of the target proteins. With methyl-bes-tatin (MeBS) as a ligand for cellular inhibitor of apoptosis protein 1 (cIAP1) ubiquitin ligase, we have developed several SNIPER compounds targeting cellular retinoic acid-binding protein 2 (CRABP2),5,6) estrogen receptor α (ERα),5,6) and spindle regulatory protein transforming acidic coiled-coil-3 (TACC3).5,7) The SNIPERs against CRABP2 and ERα induce cIAP1-mediated ubiquitylation and proteasomal degradation of the target proteins as intended.5,6) However, SNIPER(TACC3) requires anaphase-promoting complex/cyclosome with CDH1 (APC/C; CDH1) ubiquitin ligase instead of cIAP1 to induce ubiquitylation and degradation of TACC3 protein.7)

A spindle-regulatory protein TACC3 is one of the attractive targets for cancer therapy because TACC3 is aberrantly expressed in various human cancers and its depletion leads to mitotic or postmitotic cell cycle arrest and occasionally induce apoptosis of cancer cells.8–13) We have previously shown that SNIPER(TACC3) induces apoptosis selectively in cancer cells expressing a large amount of TACC3 protein as compared with normal cells.14) However, caspase activation was not extensively observed in the SNIPER(TACC3)-treated cancer cells, suggesting the involvement of different cell death mechanisms.

Cell death is classified into several types, such as apoptosis, necrosis (necroptosis), autophagy-associated death, mitotic catastrophe, paraptosis and oncosis.14) Paraptosis has been characterized by cytoplasmic vacuolization that associates with extensive swelling of endoplasmic reticulum (ER), and mitochondria in some cases, in the absence of typical apoptotic features such as formation of apoptotic bodies and nuclear fragmentation.15) Several reports have shown that paraptosis or paraptosis-like cell death (PLCD) is induced by overexpression of oncogenic genes,16,17) mutation or deletion of genes related to ER function,18–20) and exposure to natural or synthetic compounds.21–26) Notably, paraptosis or PLCD have been frequently observed in a variety of cancer cells but not in normal cells, therefore induction of paraptosis could be an alternative approach to kill cancer cells that are resistant to undergo apoptosis.21,22,24,26) In this study, we demonstrate that SNIPER(TACC3) induces cytoplasmic vacuolization and...
PLCD in cancer cells. We also showed that the SNIPER (TACC3) sensitizes cancer cells to bortezomib.

Materials and Methods

Chemical compounds. SNIPER(TACC3)-1 and -2 were synthesized as described previously. The chemical synthesis and physicochemical data on the other compounds are provided in the Doc. S1, Schemes S1 and S2.

Cell culture. Human osteosarcoma U2OS, human fibrosarcoma HT1080, and human fetal lung fibroblast TIG1, TIG3, MRC5, MRC9 were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS) and 100 μg/mL of kanamycin (Sigma, St. Louis, MO, USA). Human breast carcinoma MCF-7, human myeloid leukemia K562, and human Burkitt’s lymphoma Raji cells were maintained in RPMI 1640 medium containing 10% FBS and 100 μg/mL of kanamycin. Human multiple myeloma RPMI-8226 and KMS-11 cells were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan) Cell Bank (JCRB0034 and JCRB1179), and these cells were maintained in RPMI 1640 medium containing 10% FBS and 100 μg/mL of kanamycin.

siRNA Transfection. Cells were transiently transfected with a gene-specific short interfering RNA (siRNA) or a negative control siRNA (Qiagen, Valencia, CA, USA) using lipofectamine RNAi MAX reagent (Life Technologies, Carlsbad, CA, USA). The siRNA sequence used in this study were:

- UBE1 (5'-GGCCCAAAUAGAAUCCACU-3');
- XBPI-1 (5'-GGAGAGACCUGAAGAGAU-3');
- XBPI-2 (5'-CCUCUCUGCCUUGGUAAAG-3');
- ATF4-1 (5'-GCCUAGCUCUCUAGAUGA-3');
- ATF4-2 (5'-CCACGUUGGAGACACUUG-3');
- CHOP (5'-GCAAGCGCAUGAGAAGAAA-3');
- cIAP1-1 (5'-UCUAGAGCAGUAGAGAUCUU-3');
- cIAP1-2 (5'-GCUGAGCGUUAUACGAAUCGG-3');
- XIAP-1 (5'-ACACUGCCAGCGCAGGGUUUUCU-3');
- XIAP-2 (5'-GAAGAGUACCGGUGUGGCUUAU-3');

Western blotting. Cells were lysed with SDS lysis buffer (0.1 M Tris·HCl at pH 8.0, 10% glycerol, 1% SDS) and immediately boiled for 10 min to obtain clear lysates. The protein concentration was measured by BCA method (Pierce, Rockford, IL, USA) and the lysates containing an equal amount of proteins were separated by SDS-PAGE, transferred to PVDF membranes (Millipore, Darmstadt, Germany) for western blot analysis using the appropriate antibodies. The amount of proteins were separated by SDS-PAGE, transferred to PVDF membranes (Millipore, Darmstadt, Germany) for western blot analysis using the appropriate antibodies. The immunoreactive proteins were visualized using the Immobilon western blotting analyzer (Fuji, Tokyo, Japan). The antibodies used in this study were: ATF4 (Cell Signaling Technology, 11815, Danvers, MA, USA), CHOP (Santa Cruz, sc-7160; Cell signaling Technology, 2042), LC3 (Sigma; L7543, MBL, Nagoya, Japan; M115-3), XBP-1 (Santa Cruz, sc-7160; Cell signaling Technology, 12782), UBE1 (Cell Signaling Technology, 4890), Hsp90 (BD Biosciences, 555803), PMP70 (Abcam, Cambridge, UK; ab85550), GM130 (Abcam, ab52649), COX IV (Cell Signaling, 4850).

Cell viability assay. Cell viability was determined using water-soluble tetrazolium WST-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) for the spectrophotometric assay according to the manufacturer’s instructions (Dojindo, Tokyo, Japan). Cells treated with compounds were incubated with WST-8 reagent for 0.5 h at 37°C in a humidified atmosphere of 5% CO2. The absorbance at 450 nm of the medium was measured using an EnVision Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA).

Statistical analysis. A student’s t-test was used to determine the significance of differences among the experimental groups. Values of P < 0.05 were considered significant.

Results

SNIPER(TACC3) induces cytoplasmic vacuolization in cancer cells. When human osteosarcoma U2OS cells were treated with SNIPER(TACC3)-1 and -2, the cells formed remarkable cytoplasmic vacuolization (Fig. 1a, b). SNIPER(TACC3)s contain two different ligands, MeBS for cIAP1 and KHS108 for TACC3, which are connected by linkers. Combination treatment with MeBS and KHS108 did not induce cytoplasmic vacuolization, indicating that linking the two ligands is critically needed for the induction of cytoplasmic vacuolization. To investigate which chemical structure of SNIPER(TACC3) is required for the vacuolization, we replaced the KHS108 moiety of SNIPER(TACC3) with benzoyl-amide or biotin, and the resulting compounds did not induce vacuole formation (Fig. 1b; compound 10 and 13). In addition, other SNIPERs targeting CRABP2(3) and ERα did not induce cytoplasmic vacuolization(6) (Fig. S1). We further derivatized the SNIPER (TACC3) by replacing bestatin moiety to MV1, another IAP ligand, and this compound induced vacuolization as well as SNIPER(TACC3)-1 and -2 (Fig. 1b; compound 19). However, substitution of bestatin with fluorescein isothiocyanate (FITC) lost the ability to induce vacuolization (Fig. 1b; compound 17). Notably, the compounds with the activity to induce vacuolization caused cell death (Fig 1c). These results suggest that conjugating KHS108 to IAP ligands is required for the induction of vacuolization and cell death. Hereafter, we mainly used SNIPER(TACC3)-2 in the following experiments.

SNIPER(TACC3)-2 also induced cytoplasmic vacuolization in human breast carcinoma MCF7 and human fibrosarcoma HT1080 cells, but not in normal human fibroblast TIG1, MRC5 and MRC9 cells (Fig 1d), suggesting that SNIPER(TACC3) induces cytoplasmic vacuolization selectively in cancer cells.

To investigate the origin of the vacuoles, we stained cells with a variety of organelle markers. The vacuole was not stained with LAMP2, PMP70, GM130 and COX IV, markers of lysosome, peroxisome, golgi apparatus and mitochondria, respectively, but clearly stained with ECFP-ER that has an ER localization signal (KDEL motif) (Fig. 2). These results strongly suggest that the vacuole is derived from ER.

© 2017 The Authors. Cancer Science published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.
SNIPER(TACC3) activates the X-linked inhibitor of apoptosis protein (XIAP)-mediated ubiquitylation and ER stress response selectively in cancer cells. To investigate the mechanism by which SNIPER(TACC3) induces cytoplasmic vacuolation, the effect of siRNA-mediated gene silencing was examined. The SNIPER(TACC3)-2-induced cytoplasmic vacuolation
was prevented by silencing of ubiquitin-activating enzyme 1 (UBE1) (Fig. 3a), indicating that the ubiquitylation system is required for the vacuolization. To clarify the requirement of an IAP ubiquitin ligase for the vacuolization, we also silenced IAPs. IAP antagonists, such as MV1, are known to bind to cIAP1, cIAP2 and XIAP.\(^{27-29}\) Since cIAP2 is not expressed in U2OS cells, we focused on cIAP1 and XIAP. The silencing of XIAP, but not cIAP1, suppressed cytoplasmic vacuolization by SNIPER(TACC3)-2 (Figs 3b and S2). These results suggest that the XIAP-mediated ubiquitylation is required for the SNIPER(TACC3)-induced vacuole formation.

To examine if the ubiquitylation actually occurs in the SNIPER(TACC3)-treated cells, we immunostained the cells with antibodies against ubiquitin. Immunostaining with antibodies specific to multi-ubiquitin and K48-linked ubiquitin showed a punctate signal in the SNIPER(TACC3)-treated U2OS cells, suggesting that SNIPER(TACC3)-2 induces ubiquitylated protein aggregates in cytoplasm of U2OS cells (Fig. 4a). The ubiquitylated protein aggregates were not observed in normal fibroblasts TIG3 and MRC5 (Fig. 4b), indicating the selectivity to cancer cells. We then investigated the role of E1 and XIAP in the SNIPER(TACC3)-induced ubiquitylated protein aggregate formation. The silencing of UBE1 prevented the SNIPER(TACC3)-induced accumulation of ubiquitylated protein aggregates (Fig. 4c), which is consistent with the suppression of cytoplasmic vacuolization induced by SNIPER(TACC3) (Fig. 3a). In addition, XIAP silencing reduced the SNIPER(TACC3)-induced accumulation of ubiquitylated proteins (Fig. 4d), indicating that XIAP-mediated ubiquitylation takes place in the SNIPER(TACC3)-treated cells.

Since accumulation of ubiquitylated protein aggregates often causes ER stress in a variety of cell system,\(^{30,31}\) we next examined ER stress responses in the SNIPER(TACC3)-treated cells. Consistent with the accumulation of ubiquitylated protein aggregates, SNIPER(TACC3)-2 induced the expression of activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP), and the spliced form of X-box binding protein-1 (XBP-1), mediators of ER-stress response, in U2OS cells, which were suppressed by E1 silencing (Fig. 3a). However, the ER stress responses were not observed in normal fibroblasts, TIG3 and MRC5 (Fig. 5a). To examine which ER-stress response pathway mediates the SNIPER(TACC3)-induced cytoplasmic vacuolization, we silenced ATF4, CHOP and XBP-1 in the cells. The silencing of XBP-1 repressed the
Fig. 3. Requirement of UBE1 and XIAP for the SNIPER(TACC3)-induced cytoplasmic vacuolization. (a) UBE1 silencing represses cytoplasmic vacuolization. U2OS cells were transfected with the indicated siRNA for 24 h and treated with 30 μM SNIPER(TACC3)-2 for 5 h. Phase-contrast images were obtained. (left). Scale Bars: 20 μm. Whole cell lysates were analyzed by western blotting with the indicated antibodies (right). (b) XIAP silencing represses cytoplasmic vacuolization. U2OS cells were transfected with the indicated siRNA for 24 h and treated with 15 μM SNIPER(TACC3)-2 for 18 h. Phase-contrast images were obtained (left). Whole cell lysates were analyzed by western blotting with the indicated antibodies (right).

Fig. 4. SNIPER(TACC3) induces accumulation of ubiquitylated protein aggregates in cancer cells. (a, b) SNIPER(TACC3) induces accumulation of ubiquitylated proteins in cancer cells. Cells were treated with 30 μM SNIPER(TACC3)-2 for 4 h, and fixed and stained with the indicated anti-ubiquitin specific antibodies and Hoechst 33342. Images were obtained by fluorescent microscopy. Scale Bars: 20 μm. (c) UBE1 silencing represses the SNIPER(TACC3)-induced accumulation of ubiquitylated proteins. U2OS cells were transfected with the indicated siRNA for 24 h and treated with 30 μM of SNIPER(TACC3)-2 for 3 h. Cells were fixed and stained with indicated anti-multi-ubiquitin antibody and Hoechst 33342. Scale Bars: 20 μm. (d) XIAP silencing represses the SNIPER(TACC3)-induced accumulation of ubiquitylated proteins. U2OS cells were transfected with the indicated siRNA for 24 h and treated with 15 μM of SNIPER(TACC3)-2 for 18 h. Cells were fixed and stained with indicated anti-multi-ubiquitin antibody. Scale Bars: 20 μm.
SNIPER(TACC3)-2-induced vacuolization, while ATF4 and CHOP silencing did not (Fig. 5b), suggesting that XBP-1 plays an important role in mediating vacuole formation induced by SNIPER(TACC3)-2. Cell death characterized by ER vacuolization accompanied by ER stress response and accumulation of ubiquitylated protein aggregates is known as paraptosis or PLCD(15,16), and this type of cell death is often suppressed by a protein synthesis inhibitor and thiol antioxidants.(16,21–26) Consistent with these reports, the SNIPER(TACC3)-2-induced cytoplasmic vacuolation is inhibited by a protein synthesis inhibitor, cycloheximide (CHX) or thiol antioxidants, N-Acetyl-L-cysteine (NAC) and N-(2-Mercaptopyrrolonyl)glycine (NMPG). U2OS cells were treated with 50 μg/mL CHX, 20 mM NAC or NMPG in the presence of 30 μM SNIPER(TACC3)-2 for 5 h. Phase-contrast images were obtained. Scale Bars: 20 μm. (d) U2OS cells were treated with 20 μg/mL CHX, 10 mM NAC or NMPG in the presence or absence of 20 μM SNIPER(TACC3)-2 for 24 h and cell viability was measured by WST-8 cell proliferation assay. The graphs show the means ± SD of a representative experiment performed in triplicate; asterisks indicate P < 0.05 compared with SNIPER(TACC3)-2 alone.

Cell death characterized by ER vacuolization accompanied by ER stress response and accumulation of ubiquitylated protein aggregates is known as paraptosis or PLCD,15,16 and this type of cell death is often suppressed by a protein synthesis inhibitor and thiol antioxidants.15,16,21–26 Consistent with these reports, the SNIPER(TACC3)-2-induced cytoplasmic vacuolization and cell death were also inhibited by co-treatment with cycloheximide (CHX) and thiol antioxidants, N-acetylcysteine (NAC) and N-(2-mercaptoethyl)glycine (NMPG) (Fig. 5c,d). Necrosis (necroptosis) and oncosis also represent cell death with ER vacuolization, however, these types of cell death are not inhibited by CHX treatment.15,16,32,33 Collectively, these results strongly suggest that SNIPER(TACC3) induces the accumulation of ubiquitylated protein aggregates mediated by XIAP, which causes ER stress and vacuole formation culminating in PLCD of cancer cells.

**Combination of SNIPER(TACC3) and bortezomib.** Bortezomib and MG132 induce ER stress by inhibiting proteasome, therefore, we next examined the combination of these drugs with SNIPER(TACC3) on the vacuole formation. As shown in Figure 6a, MG132 and bortezomib at 1 μM did not induce the vacuolization. However, they enlarged the size of vacuoles induced by 30 μM SNIPER(TACC3)-2. They also induced vacuole formation when combined with 5, 10 and 20 μM of SNIPER(TACC3)-2 that scarcely induced vacuole formation by single treatments (Fig. 6a). These results suggest that proteasome inhibition enhances the SNIPER(TACC3)-induced vacuolization.

Since bortezomib is clinically used to treat multiple myelomas,34 we then evaluated the anticancer effects of SNIPER(TACC3)-2 in cancer cells including human multiple myeloma RPMI-8226 and KMS-11 cells, human Burkitt’s lymphoma
Raji cells and U2OS cells. The viability of these cancer cells were reduced by individual treatment of bortezomib and SNIPER(TACC3) in a dose-dependent manner (Fig. 6b,c). When these cancer cells were co-treated with suboptimal doses of bortezomib and SNIPER(TACC3), the viability was synergistically reduced (Fig. 6d). Thus, SNIPER(TACC3) sensitizes cancer cells to bortezomib.

**Discussion**

In our previous study, we developed SNIPER(TACC3)s that induces degradation of TACC3 protein via the ubiquitin-proteasome pathway. The SNIPER(TACC3) caused significant apoptosis in cancer cells expressing larger amount of TACC3 protein, however, caspase activation was not extensively observed. To understand how SNIPER(TACC3) induces cell death in cancer cells in more detail, we analyzed the SNIPER(TACC3)-treated cancer cells, and found that SNIPER(TACC3) also induces cytoplasmic vacuolization and PLCδ selectively in cancer cells (Fig. 7). The SNIPER(TACC3)-induced degradation of TACC3 is important for induction of apoptosis in cancer cells. However, the down-regulation of TACC3 is not likely to be involved in the induction of PLCδ because the vacuolization was not blocked by siRNA-mediated knockdown of APC/C(Cdh1)-components that plays an important role in the TACC3 degradation by SNIPER(TACC3) (Fig. S3) and siRNA-mediated knockdown of TACC3 did not induce cytoplasmic vacuolization (Fig. S4).

The vacuolization and PLCδ were induced by both bestatin-based and MV-1-based SNIPER(TACC3)s, but substitution of the IAP-ligands to FITC abolished the activity, suggesting that IAPs play a role in the SNIPER(TACC3)-induced vacuolization. In line with this, silencing of XIAP suppressed the SNIPER(TACC3)-induced accumulation of the ubiquitylated protein aggregates and cytoplasmic vacuolization, indicating an indispensable role of XIAP in this process. Thus, SNIPER(TACC3) induces XIAP-mediated ubiquitylation, which results in the accumulation of ubiquitylated protein aggregates. Since the ubiquitylated protein aggregates often cause cytoplasmic vacuolization, the SNIPER(TACC3)-induced accumulation of ubiquitylated aggregates would results in the vacuolization as well. At present, the ubiquitylated proteins...
included in the aggregates were not clear. TACC3 was not found in the aggregates (data not shown) probably because most of the TACC3 protein is degraded in the cells.

Although the ubiquitylated proteins are not identified, the protein aggregates accumulated in the SNIPER(TACC3)-treated cells would cause ER-stress. Substantial and prolonged accumulation of misfolded proteins in the ER would bring about extensive ER swelling. XBP-1 was reported to induce ER expansion and ER biogenesis.\(^{18,39}\) Consistent with these reports, the vacuoles induced by SNIPER(TACC3) are derived from ER, and the vacuolization requires expression of XBP-1. This evidence suggests that the suppression of SNIPER(TACC3)-induced vacuolization by CHX (Fig. 5c) could be attributed to the inhibition of XBP-1 expression (Fig. S5).

The SNIPER(TACC3)-induced cytoplasmic vacuolization and PLCD were observed in cancer cells but not in normal fibroblasts. Since cancer cells accumulate mutations in their genome, they produce more amounts of abnormal mutant proteins than normal cells, and therefore, cancer cells are constantly under an ER-stressed condition.\(^{40}\) In addition, new protein synthesis and thiol homeostasis are more essential for rapidly growing cancer cells than quiescent normal cells.\(^{21,22,41}\) This evidence could explain, at least in part, why SNIPER(TACC3) showed selectivity to cancer cells. In addition, the expression level of XIAP might be involved in the selectivity to cancer cells, because XIAP is overexpressed in a variety of cancer cells.\(^{42,43}\)

Bortezomib enhanced the SNIPER(TACC3)-induced vacuolization, and the combination treatment of bortezomib and SNIPER(TACC3) exhibited a synergistic anticancer activity against several cancer cell lines. Since SNIPER(TACC3) impedes ER-associated protein degradation (ERAD) system, a target of proteasome inhibitors, and induces ER stress responses, the susceptibility of cancer cells to bortezomib could be enhanced by SNIPER(TACC3). Combination of these would be effective to treat a variety of cancers represented by multiple myelomas, in which the ER is expanded to accommodate the synthesis of secretory immunoglobulin.\(^{44}\) Meanwhile, the SNIPER(TACC3)-induced PLCD was mediated by XIAP as described above, which is involved in the resistance to cancer therapy by inhibiting apoptosis.\(^{42,43}\) Therefore, the induction of PLCD by SNIPER(TACC3) might be applied to treat cancers resistant to apoptosis by overexpression of XIAP.

Acknowledgments
The authors appreciate Dr. K. Okuhira for helpful discussions. We thank Dr. M. Tada for kindly providing CFP-ER plasmid. This work was supported in part by grants from Japan Society for the Promotion of Science (KAKENHI Grants 26860049 to N.O.; 16H05090 and 16K15121 to M.N.), Japan Agency for Medical Research and Development (AMED) (15ak010129h1402 and 16ak010129j1403 to M.N.; 16cm0106124j0001 to N.O.), Ministry of Health and Labour Welfare (to M.N) and Takeda Science Foundation (to N.O.).

Disclosure Statement
K. Nagai, H. Nara and N. Cho are employees of Takeda Pharmaceutical Co., Ltd. M. Naito received a research fund from Takeda.

\[\text{Fig. 7. Possible schematic model of SNIPER (TACC3)-induced death in cancer cells. SNIPER (TACC3) induces APC/C^{Cdh1}-mediated ubiquitylation and proteasomal degradation of TACC3 protein, which results in activation of caspases and apoptosis in some cancer cells (right pathway). SNIPER(TACC3) also induces XIAP-mediated ubiquitylation and causes accumulation of ubiquitylated protein aggregates, resulting in cytoplasmic vacuolization. Then, a large population of cancer cells appears to undergo PLCD (left pathway).}\]
References

1 Ohoka N, Shibata N, Hattori T, Naito M. Protein knockdown technology: application of ubiquitous ligase to cancer therapy. Curr Cancer Drug Targets 2016; 16: 136–46.

2 Itoh Y, Ishikawa M, Naito M, Hashimoto Y. Protein knockdown using methyl benzin-ligand hybrid molecules: design and synthesis of inducers of ubiquitin-mediated degradation of cellular retinoid acid-binding proteins. J Am Chem Soc 2010; 132: 5820–6.

3 Okuhira K, Ohoka N, Sui K et al. Specific degradation of CRABP-II via cIAP1-mediated ubiquitylation induced by hybrid molecules that crosslink cIAP1 and the target protein. FEBS Lett 2011; 585: 1147–52.

4 Demizu Y, Okuhira K, Motiz H et al. Design and synthesis of estrogen receptor degradation inducer based on a protein knockdown strategy. Bioorg Med Chem Lett 2012; 22: 1793–6.

5 Okuhira K, Demizu Y, Hattori T et al. Molecular design, synthesis, and evaluation of SNIPER/ER) that induces proteasomal degradation of ERalpha. Methods Mol Biol 2016; 1566: 549–60.

6 Okuhira K, Demizu Y, Hattori T et al. Development of hybrid small molecules that induce degradation of estrogen receptor-alpha and necrotic cell death in breast cancer cells. Cancer Sci 2013; 104: 1492–8.

7 Ohoka N, Nagai K, Hattori T et al. Cancer cell death induced by novel small molecules degrading the TACC3 protein via the ubiquitin-proteasome pathway. Cell Death Dis 2014; 5: e1513.

8 Ma XJ, Salunga R, Tuggle JT et al. Gene expression profiles of human breast cancer progression. Proc Natl Acad Sci USA 2003; 100: 5974–9.

9 Ulisse S, Baldini E, Toller M et al. Transforming acidic coiled-coil 3 and Aurora-A interact in human thcytocytes and their expression is deregulated in thyroid cancer tissues. Endocr Relat Cancer 2007; 14: 827–37.

10 Peters DG, Kudla DM, Deloa IA et al. Comparative gene expression analysis of ovarian carcinoma and normal ovarian epithelium by serial analysis of gene expression. Cancer Epidemiol Biomarkers Prev 2005; 14: 1717–23.

11 Kiemeney LA, Sulem P, Besenbacher S et al. A sequence variant at 4p16.3 confers susceptibility to urinary bladder cancer. Nat Genet 2010; 42: 415–19.

12 Yao R, Kondoh Y, Natsume Y et al. Functional ATPase activity of cIAP1 and the target protein. FEBS Lett 2014; 582: 1147–52.

13 Ohoka N, Shibata N, Hattori T et al. Protein knockdown technology: application of ubiquitous ligase to cancer therapy. Curr Cancer Drug Targets 2016; 16: 136–46.

14 Kar R, Singh PK, Venkatachalam MA, Saikumart P. A novel role for MALP LC5 in non-augmophagic cytoplasmatic vacuation death of cancer cells. Oncogene 2009; 28: 2556–68.

15 Singh PK, Pandeswara S, Venkatachalam MA, Saikumart P, Manumycin A inhibits triple-negative breast cancer growth through LC3-mediated cytoplasmatic vacuolation death. Cell Death Dis 2013; 4: e457.

16 Wang WB, Feng LX, Yue QX et al. Paraptosis accompanied by autophagy and apoptosis was induced by celastrol, a natural compound with influence on proteasome, ER stress and Hsp90. J Cell Physiol 2012; 227: 2196–206.

17 Wasik AM, Almestrand S, Wang X et al. WINS5,212-2 induces cytoplasm vacuolation in apoptosis-resistant MCL cells. Cell Death Dis 2011; 2: e225.

18 Tardito S, Isella C, Medico E et al. The thixotrozirole copper(II) complex A0 induces endoplasmatic reticulum stress and paraptotic death in human cancer cells. J Biol Chem 2009; 284: 24306–19.

19 Yoon MJ, Kang YJ, Lee JA et al. Stronger proteasome inhibition and higher CHOP induction are responsible for more effective induction of paraptosis by dihydroxycurcumin than curcumin. Cell Death Dis 2014; 5: e1112.

20 Varfolomeev E, Blankenship JW, Wayson SM et al. IAP antagonists induce autoubiquitiniation of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell 2007; 131: 669–83.

21 Vinc JE, Wong WW, Khan N et al. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 2007; 131: 682–93.

22 Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X, Harren PG. A small molecule Smac mimetic potentiates TRAIL- and TNFalpha-mediated cell death. Science 2004; 305: 1471–4.

23 Bence NF, Sampat RM, Kopito RR. Impairment of the ubiquitin-proteasome system by protein aggregation. Science 2001; 292: 1552–5.

24 Rao RV, Bredesen DE. Misfolded proteins, endoplasmatic reticulum stress and neurodegeneration. Curr Opin Cell Biol 2004; 16: 653–62.

25 Hallock S, Tang SC, Baja LM, Trump BF, Liepins A, Weaersaging P. Aurintricarbonyl acid induces protein synthesis independent, sarginuin- induced apoptosis and oncosis. Toxicol Pathol 2006; 35: 300–9.

26 Suarez Y, Gonzalez L, Berciano M, Lafarga M, Munoz A. Intracellular vacuolization death. Phil Trans R Soc B 2011; 366: 98–109.

27 Wasik AM, Almestrand S, Wang X et al. WINS5,212-2 induces cytoplasm vacuolation in apoptosis-resistant MCL cells. Cell Death Dis 2011; 2: e225.

28 Vinc JE, Wong WW, Khan N et al. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 2007; 131: 682–93.

29 Yoon MJ, Kang YJ, Lee JA et al. Stronger proteasome inhibition and higher CHOP induction are responsible for more effective induction of paraptosis by dihydroxycurcumin than curcumin. Cell Death Dis 2014; 5: e1112.

30 Varfolomeev E, Blankenship JW, Wayson SM et al. IAP antagonists induce autoubiquitiniation of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell 2007; 131: 669–83.

31 Vinc JE, Wong WW, Khan N et al. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 2007; 131: 682–93.

32 Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X, Harren PG. A small molecule Smac mimetic potentiates TRAIL- and TNFalpha-mediated cell death. Science 2004; 305: 1471–4.

33 Bence NF, Sampat RM, Kopito RR. Impairment of the ubiquitin-proteasome system by protein aggregation. Science 2001; 292: 1552–5.

34 Rao RV, Bredesen DE. Misfolded proteins, endoplasmatic reticulum stress and neurodegeneration. Curr Opin Cell Biol 2004; 16: 653–62.

35 Hallock S, Tang SC, Baja LM, Trump BF, Liepins A, Weaersaging P. Aurintricarbonyl acid induces protein synthesis independent, sarginuin- induced apoptosis and oncisis. Toxicol Pathol 2006; 35: 300–9.

36 Suarez Y, Gonzalez L, Berciano M, Lafarga M, Munoz A. Intracellular vacuolization death. Phil Trans R Soc B 2011; 366: 98–109.

37 Wasik AM, Almestrand S, Wang X et al. WINS5,212-2 induces cytoplasm vacuolation in apoptosis-resistant MCL cells. Cell Death Dis 2011; 2: e225.

38 Vinc JE, Wong WW, Khan N et al. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 2007; 131: 682–93.

39 Yoon MJ, Kang YJ, Lee JA et al. Stronger proteasome inhibition and higher CHOP induction are responsible for more effective induction of paraptosis by dihydroxycurcumin than curcumin. Cell Death Dis 2014; 5: e1112.

40 Varfolomeev E, Blankenship JW, Wayson SM et al. IAP antagonists induce autoubiquitiniation of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell 2007; 131: 669–83.

41 Vinc JE, Wong WW, Khan N et al. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 2007; 131: 682–93.

42 Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X, Harren PG. A small molecule Smac mimetic potentiates TRAIL- and TNFalpha-mediated cell death. Science 2004; 305: 1471–4.

43 Bence NF, Sampat RM, Kopito RR. Impairment of the ubiquitin-proteasome system by protein aggregation. Science 2001; 292: 1552–5.

44 Rao RV, Bredesen DE. Misfolded proteins, endoplasmatic reticulum stress and neurodegeneration. Curr Opin Cell Biol 2004; 16: 653–62.

45 Hallock S, Tang SC, Baja LM, Trump BF, Liepins A, Weaersaging P. Aurintricarbonyl acid induces protein synthesis independent, sarginuin- induced apoptosis and oncisis. Toxicol Pathol 2006; 35: 300–9.

46 Suarez Y, Gonzalez L, Berciano M, Lafarga M, Munoz A. Intracellular vacuolization death. Phil Trans R Soc B 2011; 366: 98–109.
Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. SNIPER (CRABP2) does not induce cytoplasmic vacuolization.

Fig. S2. Phase-contrast images of XIAP- and cIAP1-silenced cells treated without SNIPER(TACC3), corresponding to Fig. 3b.

Fig. S3. APC3 and CDH1 silencing represses the degradation of TACC3, but not the vacuolization, induced by SNIPER(TACC3).

Fig. S4. TACC3 silencing does not induce cytoplasmic vacuolization.

Fig. S5. CHX, but not NAC and NMPG, inhibits the SNIPER(TACC3)-induced XBP1 expression.

Doc. S1. The chemical synthesis and physicochemical data.

Scheme S1. Synthesis of the compounds 10 and 13.

Scheme S2. Synthesis of the compounds 17 and 19.

44 Leung-Hagesteijn C, Erdmann N, Cheung G et al. Xbp1s-negative tumor B cells and pre-plasmablasts mediate therapeutic proteasome inhibitor resistance in multiple myeloma. *Cancer Cell* 2013; 24: 289–304.

45 Berenbaum MC. Criteria for analyzing interactions between biologically active agents. *Adv Cancer Res* 1981; 35: 269–335.