Derivatization of inhibitor of apoptosis protein (IAP) ligands yields improved inducers of estrogen receptor α degradation

Nobumichi Ohoka1, Yoko Morita2,3, Katsunori Nagai2,3, Kenichiro Shimokawa2, Osamu Ujikawa2,3, Ikuo Fujimori2, Masahiro Ito2, Youji Hayase2, Keiichiro Okuhira1,4, Norihiro Shibata1, Takayuki Hattori1, Tomoya Sameshima2, Osamu Sano2, Ryokichi Koyama2,5, Yasuhiro Imaeda2, Hiroshi Nara2,6, Nobuo Cho2,7, Mikihiko Naito1,*

From the 1 Division of Molecular Target and Gene Therapy Products, National Institute of Health Sciences, Kanagawa 210-9501, Japan
2 Pharmaceutical Research Division, Takeda Pharmaceutical Co. Ltd., Kanagawa 251-8555, Japan

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3 Present address: Axcelead Drug Discovery Partners, Inc., Kanagawa 251-8555, Japan
4 Present address: Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima 770-8505, Japan
5 Present address: SCOHIA PHARMA, Inc., Kanagawa 251-8555, Japan
6 Present address: The Pharmaceutical Society of Japan, Tokyo 150-0002, Japan
7 Present address: Drug Discovery Chemistry Platform Unit (Wako branch), RIKEN Center for Life Science Technologies, Saitama 351-0198, Japan

* To whom correspondence should be addressed: Mikihiko Naito, Division of Molecular Target and Gene Therapy Products, National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki, Kanagawa 210-9501, Japan
TEL: +81 44 270 6533; Fax: +81 44 270 6534; E-mail: miki-naito@nihs.go.jp

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ABSTRACT Aberrant expression of proteins often
Development of potent SNIPER derivatives against ER\textsubscript{α} underlies many diseases, including cancer. A recently developed approach in drug development is small molecule–mediated, selective degradation of dysregulated proteins. We have devised a protein-knockdown system that utilizes chimeric molecules termed specific and nongenetic IAP-dependent protein erasers (SNIPERs) to induce ubiquitylation and proteasomal degradation of various target proteins. SNIPER(ER)-87 consists of an inhibitor of apoptosis protein (IAP) ligand LCL161 derivative that is conjugated to the estrogen receptor \textalpha (ER\textalpha) ligand 4-hydroxy-tamoxifen by a PEG linker, and we have previously reported that this SNIPER efficiently degrades the ER\textalpha protein. Here, we report that derivatization of the IAP ligand module yields SNIPER(ER)s with superior protein-knockdown activity. These improved SNIPER(ER)s exhibited higher binding affinities to IAPs and induced more potent degradation of ER\textalpha than does SNIPER(ER)-87. Further, they induced simultaneous degradation of cellular inhibitor of apoptosis protein 1 (cIAP1) and delayed degradation of X-linked IAP (XIAP). Notably, these reengineered SNIPER(ER)s efficiently induced apoptosis in MCF-7 human breast cancer cells that require IAPs for continued cellular survival. We found that one of these molecules, SNIPER(ER)-110, inhibits the growth of MCF-7 tumor xenografts in mice more potently than the previously characterized SNIPER(ER)-87. Mechanistic analysis revealed that our novel SNIPER(ER)s preferentially recruit XIAP, rather than cIAP1, to degrade ER\textalpha. Our results suggest that derivatized IAP ligands could facilitate further development of SNIPERs with potent protein-knockdown and cytocidal activities against cancer cells requiring IAPs for survival.

Aberrant or increased expression of pathogenic proteins often results in the initiation of many diseases, including a variety of cancers. However, a limited number of pathogenic proteins are currently targeted by pharmacological inhibitors, primarily because many proteins do not have suitable binding pockets where a pharmacophore may interact to regulate protein activity (1,2). An alternative approach has been to down-regulate expression of the pathogenic proteins; this is usually achieved \textit{in vitro} through use of genetic methods involving antisense oligonucleotides, double-stranded RNAs and CRISPR-Cas9 technology. However, clinical application of these technologies remains challenging because delivery of
oligonucleotides to the target tissues is not easily accomplished (3,4).

As a novel strategy to down-regulate pathogenic proteins in a non-genetic manner, we and others have devised a protein knockdown system that uses small molecules with sufficient membrane permeability to induce selective degradation of target proteins. These small molecule compounds, designated as Proteolysis Targeting Chimeras (PROTACs) and Specific and Non-genetic IAP-dependent Protein Erasers (SNIPERs), are chimeric molecules that contain two different ligands connected by a linker—one ligand is specific for an E3 ubiquitin ligase and the other is specific for a target protein (5-7). The PROTACs and SNIPERs are designed to cross-link the E3 ubiquitin ligase and the target protein to induce polyubiquitylation and proteasomal degradation of the target protein within cells. To recruit the von Hippel-Lindau (VHL) E3 ligase complex and the cereblon (CRBN) E3 ligase complex, a VHL inhibitor (based on the HIF-1α peptide) and a phthalimide moiety have been respectively integrated into PROTAC constructs (8-11). In a similar manner, an IAP antagonist has been incorporated into SNIPERs to recruit either cellular inhibitor of apoptosis protein 1 (cIAP1) or X-linked inhibitor of apoptosis protein (XIAP) E3 ligase (12-18). To date, a range of PROTAC and SNIPER compounds have been developed, allowing degradation of a variety of proteins, such as estrogen receptor α (ERα), oncogenic kinase BCR-ABL, and epigenetic regulator bromodomain-containing protein 4 (BRD4). (19-30). Some PROTACs and SNIPERs also have demonstrated the ability to degrade target proteins in vivo, suggesting that this technology is feasible for use in novel drug development. These include VHL-based and CRBN-based PROTACs directed against BRD4, which are capable of inducing BRD4 protein degradation in mouse xenograft models (10,31). By incorporating an LCL161 derivative, a high-affinity IAP ligand, we developed SNIPER(ER)-87, which induces in vivo degradation of ERα and growth inhibition of an ERα-positive human breast tumor in a xenograft model (13).

IAPs are a family of antiapoptotic proteins containing one or three baculoviral IAP repeat (BIR) domains (32-34). Some family members, such as cIAP1, cIAP2, and XIAP, directly interact with and regulate caspases via the BIR domain, thus inhibiting apoptosis (35-38). These IAPs are attractive targets for tumor therapy because of their frequent overexpression in multiple human malignancies and their implications in tumor progression, treatment failure, and poor prognosis (39-45). Based on the IAP-binding
Development of potent SNIPER derivatives against ERα tetrapeptides of second mitochondria-derived activator of caspases/direct IAP binding protein with low pl (SMAC/DIABLO), many potent and cell-permeable peptidomimetic IAP antagonists (also known as SMAC mimetics) have been developed; some of these are under evaluation in clinical phase studies as antitumor drugs (32,46,47). These IAP antagonists interact with BIR domains in IAP proteins to directly inhibit XIAP or to induce autoubiquitylation and proteasomal degradation of cIAP1 and cIAP2 (48-51). Since SNIPERs utilize IAP antagonists as IAP-ligand modules, SNIPERs are able to downregulate IAPs beyond the initial target proteins (12-18); this is likely to be advantageous when attempting to kill cancer cells that require IAPs for survival.

In this paper, we demonstrate that, by derivatizing the IAP-ligand module, we have developed novel SNIPER(ER)s whose protein knockdown and antitumor activities are more potent than those of SNIPER(ER)-87. These SNIPER(ER)s have higher affinity for IAPs and exhibit more consistent abilities to degrade ERα and IAPs. In addition, we discuss the significance of IAP downregulation in pharmacological efforts to induce cancer cells to undergo apoptosis.

RESULTS

Structure-activity relationship of SNIPER(ER)s with novel IAP ligands.

To discover IAP ligands that are useful for the development of SNIPERs with potent protein knockdown activity, we substituted the IAP ligand moiety of SNIPER(ER)-87 with several IAP antagonists that have been reported elsewhere (52-55) (Fig. 1 and Table 1), and examined their abilities to reduce ERα expression by MCF-7 human breast tumor cells (Fig. 2). In a series of SNIPER(ER)s with different IAP ligand modules, we found that five compounds (SNIPER(ER)-105 (4), -110 (31a), -113 (31c), -119 (31d), -126 (50)) reduced ERα expression comparably to, and more potently than, SNIPER(ER)-87 for durations of 4 h and 48 h, respectively (Fig. 2a). SNIPER(ER)-130 (69) and -131 (71) exhibited attenuated knockdown activities, while the others (SNIPER(ER)-104 (9), -118 (55a), -121 (55b), -134 (31b), -136 (40)) did not induce effective knockdown at 1-100 nM (Fig. 1b). The DC_{50} (the concentration required for 50% reduction of expression) for each SNIPER(ER) compound is summarized in Table 1.

Table 1 also reports the binding affinities (IC_{50}) of SNIPER(ER)s to ERα and IAPs in vitro. Since SNIPER(ER)-87 preferentially recruits XIAP to degrade ERα (13), we compared the binding affinity to XIAP with
the DC\textsubscript{50}, for all SNIPER(ER)s. SNIPER(ER)s with effective knockdown activities (SNIPER(ER)-105, -110, -113, -119, -126) exhibited affinities to XIAP that were greater than, or comparable to, SNIPER(ER)-87; in contrast, those with little or no ER\textalpha{} knockdown activity (SNIPER(ER)-104, -118, -121, -134, -136) exhibited lower binding affinities to XIAP. However, SNIPER(ER)-130 and -131 exhibited attenuated ER\textalpha{} knockdown activities, despite their higher affinities for XIAP binding. These results suggest that an increased binding affinity of SNIPER(ER) for XIAP seems to be important for effective ER\textalpha{} knockdown, but is not the sole determinant for degradation activity. Among the arene substitution isomers (SNIPER(ER)-110, -113, and -119), SNIPER(ER)-110 exhibited slightly better ability to degrade ER\textalpha{}; therefore, we chose SNIPER(ER)-110 as a representative. SNIPER(ER)-105, -110 and -126 also showed more potent ability to degrade ER\textalpha{}, compared with SNIPER(ER)-87, in assays involving other human ER\textalpha{}-positive breast tumor T47D and ZR-75-1 cells (Fig. 3).

\textbf{Degradation of cIAP1 and XIAP by SNIPER(ER)s.}

As observed in studies of many IAP antagonists, SNIPERs in our study rapidly induced proteosomal degradation of cIAP1, along with degradation of their target proteins (12-18). SNIPER(ER)-105, -110, -113, -119 and -126 each reduced the cIAP1 level more potently than SNIPER(ER)-87 (Fig. 2a and 3b), consistent with their increased binding affinities for cIAP1 (Table 1). Further, these SNIPER(ER)s reduced XIAP expression in MCF-7 cells after 48 h in a more potent fashion than SNIPER(ER)-87 (Fig. 2a). The reduction of XIAP by SNIPER(ER)s was prominent in T47D cells after 48 h, but was weak after 4 h of exposure (Fig. 3b). The difference in the degradation pattern between cIAP1 and XIAP suggests that these IAPs are degraded through a different mechanism, as discussed below.

\textbf{XIAP is required for the ER\textalpha{} degradation by SNIPER(ER)s.}

We previously reported that XIAP is preferentially recruited to ER\textalpha{} upon treatment with SNIPER(ER)-87 (13). To examine which IAP is recruited by the novel SNIPER(ER)s (SNIPER(ER)-105, -110 and -126) to induce ER\textalpha{} degradation, MCF-7 and T47D cells were treated with or without the SNIPER(ER)s in the presence of MG132, a proteasome inhibitor; cell lysates were analyzed by co-immunoprecipitation. Fig. 4a shows that, when cells were treated with SNIPER(ER)s, the anti-ER\textalpha{} precipitates
contained both XIAP and cIAP1. We did not examine cIAP2, because it is not expressed in MCF-7 and T47D cells under normal cell culture and cIAP1-downregulated conditions (Supplementary Fig. S1a-c). Upon comparison of IAP protein levels in total cellular lysates, we found that SNIPER(ER)s more effectively recruited XIAP to ERα, compared with cIAP1. Consistent with this, depletion of XIAP by siRNA substantially suppressed the ERα degradation induced in MCF-7 and T47D cells by our SNIPER(ER) molecules, whereas depletion of cIAP1 marginally suppressed the degradation (Fig. 4b). These results indicate that SNIPER(ER)-105, -110 and -126 preferentially recruit XIAP to degrade ERα in target cells, similar to the mechanism initiated by SNIPER(ER)-87.

**SNIPER(ER)s induce apoptosis in tumor cells that requires IAPs for survival.**

Because ERα plays an important role in the proliferation of most primary breast tumors (56,57), we examined the effect of SNIPER(ER)s on the growth of breast tumor cells. SNIPER(ER)s and fulvestrant, a clinically-approved inducer of ERα degradation, both efficiently suppressed the growth of ERα-positive breast tumor cells (MCF-7 and T47D), but not of ERα-negative breast tumor cells (MDA-MB-231) (Fig. 5a and Supplementary Fig. S2). Notably, SNIPER(ER)-105, -110, and -126 exhibited superior anti-proliferative effects on MCF-7 cells at concentrations above 50 nM, compared with SNIPER(ER)-87 (Fig. 5a and Supplementary Fig. S2). In a microscopic analysis, we observed that substantial number of MCF-7 cells, but not T47D cells, underwent cell death upon treatment with SNIPER(ER)-105, -110 and -126 (Fig. 5b). Induction of apoptotic cell death in MCF-7 cells by SNIPER(ER)-105, -110 and -126 was confirmed by flow cytometric analysis and Annexin V/propidium iodide (PI) staining (Fig. 5c, 5d and Supplementary Fig. S3). Consistent with this, 100 nM of these SNIPER(ER)s activated caspase-7 and caspase-6 (detected by reduction of their precursor forms), which resulted in the cleavage of two caspase substrates, PARP and Lamin B1, in MCF-7 cells (Fig. 5e). These results indicate that SNIPER(ER)-105, -110 and -126 each induced apoptosis in MCF-7 cells more effectively than SNIPER(ER)-87; apoptosis was not observed in T47D cells.

To understand why these new SNIPER(ER)s effectively induce apoptosis in MCF-7 cells, but not in T47D cells, and because SNIPER(ER)-105, -110 and -126 each induced the degradation of cIAP1 and XIAP more potently than SNIPER(ER)-87.
(Fig. 2a and Fig. 3b), we investigated the effects of IAP depletion in MCF-7 and T47D cells. In MCF-7 cells, individual silencing of cIAP1 and XIAP by siRNA slightly reduced the number of cells; combined silencing of cIAP1 and XIAP reduced the number of cells by approximately 50%, compared with untreated cells (Fig. 6a), and was accompanied by caspase activation as demonstrated by cleavage of Lamin B1 and PARP (Fig. 6b). In contrast, similar silencing of IAPs in T47D cells neither reduced cell number nor activated caspases. When ERα was silenced in MCF-7 and T47D cells, the numbers of cells in both cell lines were effectively reduced without requirement for caspase activation. However, triple silencing of ERα, XIAP and cIAP1 efficiently reduced MCF-7 cell number, compared with T47D cells; further, caspase activation was observed only in MCF-7 cells. Additionally, an IAP antagonist LCL161 sensitized tumor necrosis factor (TNF) α-dependent apoptosis in MCF-7 cells, but not T47D cells (Supplementary Fig. S4). These results suggest that MCF-7 cells, but not T47D cells, require IAPs for survival; thus, IAPs could be involved in the selective induction of apoptosis in MCF-7 cells that have been exposed to SNIPER(ER)-105, -110 and -126.

**In vivo protein knockdown activities and antitumor activities of SNIPER(ER)s**

We measured the metabolic stabilities of SNIPER(ER)-87, -105, -110, -113, -119 and -126 in liver microsomes (Supplementary Table S2) and carefully selected SNIPER(ER)-110 and -126 as test compounds for in vivo comparison with SNIPER(ER)-87. When female BALB/c mice were intraperitoneally injected with SNIPER(ER)-87, -110 and -126 (30 mg/kg body weight), ERα protein levels in ovarian tissue were effectively reduced by all SNIPERs (Fig. 7a). To evaluate SNIPER(ER) knockdown activities in a tumor model, we next applied MCF-7 breast tumor xenografts to nude mice. The ERα protein levels in orthotopic tumors were reduced by 40–50% in SNIPER(ER)-treated mice, compared with those in vehicle-treated mice (Fig. 7b and 7c); SNIPER(ER)-110 significantly showed a significant increase in protein knockdown activity at 48 h, compared with SNIPER(ER)-87. Treatment with SNIPER(ER)-87, -110 and -126 each reduced cIAP1 and XIAP protein levels in tumor xenografts (Fig. 7b and 7c), consistent with the results of our in vitro analyses (Fig. 2a). Notably, SNIPER(ER)-110 again exhibited a significant increase in the ability to reduce cIAP1 and XIAP at 24 and 48 h, compared with SNIPER(ER)-87.

To demonstrate the therapeutic
significance of these findings, we also evaluated the in vivo antitumor activity of SNIPER(ER)-110, compared with SNIPER(ER)-87, in an MCF-7 tumor xenograft model. When tumor-bearing mice received daily intraperitoneal injections of SNIPER(ER)-87 or SNIPER(ER)-110 (30 mg/kg body weight) for 16 days, SNIPER(ER)-110 attenuated MCF-7 tumor progression more effectively than SNIPER(ER)-87, as shown in measurements of tumor volume and tumor weight (Fig. 8a-c). Notably, no obvious toxicities, including body weight changes, were observed during treatment (Fig. 8d). Thus, compared with SNIPER(ER)-87, SNIPER(ER)-110 showed superior antitumor activity against ERα-positive breast tumors that require IAPs for cellular survival.

**DISCUSSION**

Chemical protein knockdown technologies, utilizing chimeric molecules such as PROTACs and SNIPERs, have been recently developed to induce degradation of target proteins in cells (5-7). Our SNIPER compounds degrade cIAP1 and XIAP along with their initial target proteins, which could be advantageous in attempts to kill cancer cells because of the frequent overexpression of IAPs to suppress apoptosis in cancer cells. In this study, we developed novel SNIPER(ER)s by incorporating various IAP ligands with higher binding affinities for IAP proteins. The resulting SNIPER(ER)s (-105, -110, and -126) each induce degradation of ERα, cIAP1 and XIAP more potently than SNIPER(ER)-87, resulting in the induction of apoptosis in MCF-7 breast tumor cells. In an in vivo tumor xenograft model, the novel SNIPER(ER)s exhibited activity to reduce ERα and IAPs, that was respectively comparable to, and better than, SNIPER(ER)-87; further, the novel SNIPER(ER)-110 showed superior antitumor activity against MCF-7 tumor xenograft, compared with SNIPER(ER)-87. These results suggest that incorporating IAP ligands with higher binding affinities may be a promising strategy to develop SNIPERs with better protein knockdown and antitumor activities.

The novel SNIPER(ER)s also exhibit better protein knockdown activity in other ERα-positive breast tumor cells, compared with SNIPER(ER)-87 (Fig. 3). However, the novel SNIPER(ER)s did not exhibit improved inhibition of growth or induction of apoptosis in T47D cells (Fig. 5), which contrasts with their activities in MCF-7 cells. We speculate that the differences in sensitivity between the two cell lines derive from differing need for IAPs, because depletion of IAPs induces apoptosis in
MCF-7 cells, but not in T47D cells (Fig. 6). The role of IAPs in cancer cell survival has been suggested in many papers (32,42-45,58), and IAP antagonists have shown antitumor activity against many tumor cells including breast tumor (46,47,59). Therefore, we hypothesize that the ability of the SNIPERs to degrade IAPs could be advantageous in treatment of cancer cells, especially those dependent on the activity of IAPs. Recently, it has been reported that the different molecular signature other than IAP frequency also specifies a sensitivity to IAP antagonists in cancer cells; the mechanism depends on autocrine release of TNFα (60-64), constitutive ubiquitylation of receptor-interacting protein kinase 1 (RIP1) (61), and expression of cellular FLICE-inhibitory protein (c-FLIP) (60) and leucine-rich repeats and immunoglobulin-like domains protein 1 (LRIG1) (65). Thus, these signatures could be used to predict sensitivity to SNIPERs as well.

Although SNIPER(ER)s induce degradation of ERα, cIAP1, and XIAP, the degradation mechanism appears different for each protein. Degradation of ERα appears to require formation of a ternary complex that is composed of ERα, SNIPER, and XIAP; we suspect this because a treatment with the individual ligand, or with an structural SNIPER(ER) analog that cannot bind to IAPs, does not induce degradation of ERα (13) (Supplementary Fig. S5). The pharmacological hook effect that is observed during SNIPER(ER)-induced ERα degradation further supports the hypothesis that ternary complex formation is required (13). In contrast, the degradation of cIAP1 is triggered by binding of the IAP antagonist module to the BIR3 domain of cIAP1, thus inducing autoubiquitylation. In this case, the ternary complex is not required and no hook effect is observed (13). Notably, the degradation mechanism of XIAP remains ambiguous. Degradation of XIAP was induced by SNIPER(ER)s but not by IAP antagonists nor by the mixture of IAP antagonist and 4-hydroxytamoxifen (4-OHT) (Supplementary Fig. S5), suggesting that the ternary complex is required for XIAP degradation. Consistent with this hypothesis, XIAP degradation by SNIPER(ER)-87 was not observed in the ERα-negative MDA-MB-231 breast cancer cells (Supplementary Fig. S5). Thus, XIAP degradation seems to require the same ternary complex formation that is required for ERα degradation. However, XIAP degradation requires more time and is inefficient, compared with degradation of the ERα target protein (Fig. 2a and Fig. 3). It is likely that part of the population of XIAP in the ternary complex is subjected to
ubiquitylation and proteasomal degradation. Since XIAP degradation was more prominently observed in T47D cells than in MCF-7 and ZR-75-1 cells, we measured the expression of ERα, cIAP1 and XIAP in these cells (Supplementary Fig. S6). We found that the ratio of XIAP to ERα is 1/3 and 1/13 less in T47D cells than in MCF-7 cells and in XR-75-1 cells, respectively, suggesting that the lower expression of XIAP may increase the significance of its degradation in T47D cells. Thus, although the degradation mechanisms differ, SNIPER(ER)s induce degradation of ERα, cIAP1 and XIAP, culminating in an anti-tumor effect that is directed against breast cancer cells.

It has been reported that ERα translocates from the cytosol to the nucleus upon binding with tamoxifen (66,67). This is also observed after treatment with SNIPER(ER)s, since the relative amount of ERα increased in the nucleus, compared with the cytosol, upon treatment with SNIPER(ER)s; this was more clearly observed in the presence of MG132 (Supplementary Fig. S7a and S7b). Interestingly, the amount of XIAP increased in the nucleus upon treatment with SNIPER(ER)s; this was also more clearly observed in the presence of MG132. These results suggest that when a SNIPER(ER) penetrates a cell, it induces formation of the ternary complex composed of ERα, SNIPER(ER) and XIAP in the cytosol; ERα then translocates to the nucleus together with the bound XIAP. Thus, in the presence of SNIPER(ER), ERα interacts with XIAP sufficiently to tether XIAP in the nucleus, consistent with the preferential recruitment of XIAP, but not cIAP1, to ERα.

In summary, we have developed potent SNIPER(ER)s by incorporation of high affinity IAP ligands. These SNIPER(ER)s induce degradation of ERα and IAPs, and exhibit antitumor activity against breast tumors that require IAPs for survival. These IAP ligand modules could be utilized to develop novel SNIPERs with activity against various oncogenic proteins and enhanced antitumor capabilities.

EXPERIMENTAL PROCEDURES

Chemistry

Chemical synthesis and physicochemical data for SNIPER compounds are provided in the Supplementary Information.

Biology

Cell culture

Human breast carcinoma MCF-7, T47D, and ZR-75-1 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 100 µg/mL kanamycin. Human breast carcinoma MDA-MB-231 cells were maintained in Dulbecco’s
modified Eagle’s medium containing 10% FBS and 100 µg/mL kanamycin. All of cell lines were purchased from ATCC (Manassas, VA, USA) and their cell authentications were confirmed by morphology, karyotyping, and PCR based approaches in ATCC. Cells were treated with various concentrations of SNIPER compounds for the indicated times.

**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. Nuclear and cytoplasmic extracts were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentration was measured by the BCA method (Pierce, Rockford, IL, USA); lysates containing equal amounts of proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Darmstadt, Germany) for western blot analysis using the appropriate antibodies. Immunoreactive proteins were visualized using the Immobilon Western chemiluminescent HRP substrate (Millipore) or Clarity Western ECL substrate (Bio-Rad, Hercules, CA, USA); light emission intensity was quantified using an LAS-3000 lumino-image analyzer equipped with Image Gauge v2.3 software (Fuji, Tokyo, Japan). The antibodies used in this study were: anti-ERα rabbit monoclonal antibody (mAb) (Cell Signaling Technology, Danvers, MA, USA; 8644), anti-ERα rabbit polyclonal antibody (pAb) (Santa Cruz, Dallas, TX, USA; sc-542, sc-543), anti-cIAP1 goat pAb (R&D Systems, Minneapolis, MN, USA; AF8181), anti-cIAP1 rat mAb (Enzo Life Sciences, Farmingdale, NY, USA; 1E1-1-10), anti-β-actin mouse mAb (Sigma A5316), anti-XIAP rabbit pAb (Cell Signaling Technology, 2042), anti-GAPDH rabbit pAb (Santa Cruz, sc-25778), anti-Lamin B1 goat pAb (Santa Cruz, sc-6216), anti-Histone H3 goat pAb (Santa Cruz, sc-8654), anti-PARP rabbit pAb (Cell Signaling Technology, 9542), anti-Caspase-6 rabbit pAb (Cell Signaling Technology, 9762), anti-Caspase-7 rabbit mAb (Cell Signaling Technology, 12827).

**Measurement of binding affinities of ERα and IAPs**

The bindings between test compounds and cIAP1, cIAP2 or XIAP were determined by Time-resolved FRET (TR-FRET) assay using His-IAP proteins (XIAP, cIAP1, or cIAP2), FITC-Smac, Tb-SA and biotinylated anti-His antibody, as described previously (14). The binding between test compounds and the human ERα protein was determined using the PolarScreen™ Estrogen Receptor-α
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Competitor Assay Green (Thermo Fisher Scientific, A15882) containing recombinant ERα full-length protein, fluormone ES2 Green2 and ES2 screening buffer. Purified ERα and fluormone ES2 were diluted with assay buffer to final concentrations of 25 and 4.5 nM, respectively, and 4 µL of each dilution was added to each well of 384-well black low-volume assay plate (Greiner, 784076). Then, 2 µL of the ES2 screening buffer, containing test compounds or DMSO, was added to the well. The plate was subjected to centrifugation and incubated at room temperature for 1 hour; then, the intensity of the fluorescence polarization signal was measured by Plate reader (Envision, PerkinElmer). Wells containing ERα and Fluormone ES2 were used as a positive control, and wells containing only Fluormone ES2 were used as a negative control. IC_{50} values and 95% confidence interval were calculated by XLfit (ID Business Solutions, fit model 204) from the data expressed as % control inhibition.

Immunoprecipitation

MCF-7 or T47D cells were treated with the indicated concentrations of the indicated compounds, in combination with 10 µM MG132, for 3 h. Cells were lysed using immunoprecipitation (IP) lysis buffer (10 mM Hepes at pH 7.4, 142.5 mM KCl, 5 mM MgCl₂, 1 mM EGTA, and 0.1% Triton X-100), containing protease inhibitor cocktails, rotated for 15 min at 4°C, and centrifuged at 15,000 rpm for 10 min at 4°C to obtain the supernatants. Lysates that had been precleared with naked protein G-Sepharose were immunoprecipitated with protein G-Sepharose beads that were preincubated with anti-ERα rabbit pAb (Santa Cruz, sc-543) for 2 h at 4°C. The precipitates were washed with IP lysis buffer four times and analyzed by western blotting.

siRNA transfection

MCF-7 or T47D 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 sequences used in this study were: human ERα-1 (5′-CGACACUGCUGCGCUACACAUCAUC U-3′); ERα-2 (5′-UCACAGACACUUUGAUCCCACCUGA U-3′); ERα-3 (5′-GACCGAAGAGGAGGGAGAAUGUGA GA-3′); cIAP1-1 (5′-UCUAGAGCAGUUGAAGACAUCUCUU-3′); cIAP1-2 (5′-GCUGUAGCUUUAUCAGAAGUGUUU-3′); cIAP1-3 (5′-GCUGUAGCUUUAUUCAGAAUCUG GU-3′);
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(5′-GGAAAUGCUCCGCAACAUCUUCA-3′); XIAP-1
(5′-ACACUGCCACGACGGUGUUUCUU-3′); XIAP-2
(5′-GAAGGAGAUACCGUGCGGUGCUUA-3′); and XIAP-3
(5′-CCAGAAUGGUCAGUACAAAGUUGAA-3′).

Cell viability assay

Cell viability was evaluated by crystal violet staining. Cells were treated with graded concentrations of the SNIPER compounds for 72 h, then stained with 0.1% crystal violet (Wako, Osaka, Japan) in 1% ethanol for 15 min at room temperature. Cells were rinsed thoroughly with distilled water, then lysed in 1% SDS. The absorbance of the cell lysate at 600 nm was measured using EnVision Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA).

In vivo protein knockdown

All procedures of the animal experiments were reviewed and approved by Institutional Animal Experiment Review Committee of the National Institute of Health Sciences (NIHS) and those experiments were conducted in accordance with the guidelines approved by the Care and Use of Animals published by Institutional Animal Ethical Committee. Mice were housed in pathogen-free animal facilities at the NIHS with 12-h light/dark cycles, and were fed rodent chow and water ad libitum. For Fig. 7a, female 6-week-old BALB/c mice (Clea Japan, Tokyo, Japan) were randomized and divided into eight treatment groups (n = 3), then treated with vehicle or 30 mg/kg SNIPER(ER)s for 24 h or 48 h. For Fig. 7b and 7c, each suspension of $1 \times 10^7$ MCF-7 cells was mixed with an equal volume of Matrigel (Corning Life Sciences, Corning, NY, USA), and injected (100 µl total) into the left and right mammary fat pads of 6-week-old female BALB/c nude mice (Clea Japan). After cell inoculation, β-estradiol solution was subcutaneously injected into the neck twice at intervals of six days. Fourteen days after the last β-estradiol injection, tumor-bearing mice were randomized and divided into two or three treatment groups as follows: for Fig. 7b: (1) vehicle treatment for 24 h (n = 3); and (2) 30 mg/kg SNIPER(ER)-87 treatment for 24 h (n = 2); (3) 30 mg/kg SNIPER(ER)-110 treatment for 24 h (n = 2); and (4) 30 mg/kg SNIPER(ER)-126 treatment for 24 h (n = 2); for Fig. 7c: (1) vehicle treatment for 48 h (n = 3); (2) 30 mg/kg SNIPER(ER)-87 treatment for 48 h (n = 3); (3) 30 mg/kg SNIPER(ER)-110 treatment for 48 h (n = 3); and (4) 30 mg/kg SNIPER(ER)-126 treatment for 48 h (n = 3). Compounds were
administered via intraperitoneal injection. After the indicated treatment periods, the mice were sacrificed and tissues were excised. Total lysates from the ovaries and tumors were analyzed by western blotting.

**In vivo tumor growth inhibition**

Each suspension of $1 \times 10^7$ MCF-7 cells was mixed with an equal volume of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), and inoculated (100 µl total) into the left and right mammary fat pads of 6-week-old female Balb/c nude mice (Clea Japan) that had received an β-estradiol pellet (6 µg per day) (Innovative Research of America, Sarasota, FL, USA) under the neck skin. After four days, mice bearing tumors of approximately 100 mm$^3$ in size were randomized and divided into two groups ($n = 7$). One group served as a control for the dosing vehicle; the other groups were administered SNIPER(ER)-87 or SNIPER(ER)-110 (30 mg/kg, intraperitoneally, every 24 h), respectively. Tumor volumes were measured every two days using a caliper and calculated according to the standard formula: \((\text{length} \times \text{width}^2)/2\). At 16 days, mice were sacrificed and the tumors were weighed and excised.

**Cell cycle analysis**

After treatment, cells were gently trypsinized and washed with serum-containing medium. Cells were collected by centrifugation, then washed with PBS and fixed in 70% ice-cold ethanol for 1 h on ice. The cells were then washed, treated with 1 mg/ml RNase A for 1 h at 37°C and stained in propidium iodide (PI) solution (50 µg/ml in 0.1% sodium citrate, 0.1% NP-40). The stained cells were analyzed in a FACScan flow cytometer (Becton Dickinson).

**Measurement of apoptosis by flow cytometer**

Apoptosis was analyzed with an Annexin V-FITC Apoptosis Detection Kit (BioVision). After treatment, cells were gently trypsinized and washed with serum-containing medium. Cells were collected by centrifugation, then washed with PBS and resuspended in Binding Buffer. The cells were stained with annexin V-FITC and PI at room temperature for 5 min in the dark, according to the manufacturer’s instructions, and analyzed in a FACScan flow cytometer (Becton Dickinson, Braintree, MA).

**Statistical analysis**

Student’s $t$-test was used to evaluate differences among the experimental groups. Values of $p<0.05$ were considered significant.
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Conflict of interest

Yoko Morita, Katsunori Nagai, Kenichiro Shimokawa, Osamu Ujikawa, Ikuo Fujimori, Masahiro Ito, Youji Hayase, Tomoya Sameshima, Osamu Sano, Ryokichi Koyama, Yasuhiro Imaeda, Hiroshi Nara and Nobuo Cho are employees of Takeda Pharmaceutical Co. Ltd.

Author Contributions

N.O. and M.N. designed the experiments; Y.M., K.N., K.S., I.F., M.I., Y.H., Y.I., H.N. and N.C. designed and synthesized the compounds; N.O., K.O., N.S., T.H., T.S., O.S. and R.K. performed the experiments and analyzed the data; N.O., K.S., O.U., H.N. and M.N. wrote the manuscript; and M.N. supervised all research. All authors discussed and checked the manuscript.
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Footnotes
The abbreviations used are: 4-hydroxytamoxifen, 4-OHT; baculoviral IAP repeat, BIR; bromodomain-containing protein 4, BRD4; cellular inhibitor of apoptosis protein 1, cIAP1; cereblon, CRBN; direct IAP binding protein with low pI, DIABLO; fetal bovine serum, FBS; immunoprecipitation, IP; monoclonal antibody, mAb; National Institute of Health Sciences, NIHS; polyclonal antibody, pAb; propidium iodide, PI; Proteolysis Targeting Chimeras, PROTACs; second mitochondria-derived activator of caspases, SMAC; Specific and Non-genetic IAP-dependent Protein Erasers, SNIPERs; Time-resolved FRET, TR-FRET; von Hippel-Lindau, VHL; X-linked inhibitor of apoptosis protein, XIAP.
Table 1. Protein knockdown activities and binding affinities of SNIPER(ER)s

| Compound No. | ERα IC₅₀ (nM) | ERα IC₅₀ (nM) (95% CI) | cIAP1 (nM) | cIAP2 (nM) | XIAP (nM) (95% CI) | Originator | Reference |
|--------------|---------------|------------------------|------------|------------|-------------------|------------|-----------|
| SNIPER(ER)-87 | 83.0          | 110 (58-200)           | 450 (360-560) | 960 (600-1500) | 700 (510-960) | Novartis   | WO2008016893 |
| SNIPER(ER)-104 | >100          | ND³                    | >1000      | >1000      | >1000             | Novartis   | WO2012080260 |
| SNIPER(ER)-105 | <3            | 69 (7-670)             | 5.3 (4.6-6.1) | 7.2 (4.9-11) | 55 (38-80)      | Novartis   | WO2012080271 |
| SNIPER(ER)-110 | <3            | 120 (68-200)           | 74 (62-89) | 73 (55-97) | 330 (250-430)   | Abbott     | 52; WO2016169989 |
| SNIPER(ER)-113 | <3            | 150 (82-290)           | 85 (75-96) | 99 (58-200) | 810 (58-200)    | Abbott     | 52; WO2016169989 |
| SNIPER(ER)-118 | >100          | ND                     | 230 (73-540) | 140 (58-200) | >1000            | AstraZeneca | 53 |
| SNIPER(ER)-119 | 15.7          | 200 (73-540)           | 80 (62-100) | 48 (28-82) | 700 (490-1000)  | Abbott     | 52; WO2016169989 |
| SNIPER(ER)-121 | >100          | ND                     | ND         | >1000      | >1000            | AstraZeneca | 53 |
| SNIPER(ER)-126 | 3.7           | 83 (25-3800)           | 68 (66-77) | 200 (150-280) | 490 (310-760)  | Novartis   | WO2008016893 |
| SNIPER(ER)-130 | 36.9          | ND                     | 41 (25-65) | 68 (60-77) | 28 (21-39)      | Genentech  | 54 |
| SNIPER(ER)-131 | 33.8          | ND                     | 80 (39-160) | 25 (22-28) | 24 (18-32)      | Genentech  | 55 |
| SNIPER(ER)-134 | >100          | ND                     | 47 (15-140) | 550 (450-670) | 360 (240-540)  | Abbott     | 52 |
| SNIPER(ER)-136 | >100          | ND                     | 47 (25-89) | 890 (790-1000) | >1000          | Genentech  | 54; WO2006069063 |

³ND, not determined.
Figure Legends

Figure 1: Chemical structures of novel SNIPER(ER)s. The IAP ligand moiety of SNIPER(ER)-87 was substituted by various IAP antagonists. Chemical structures shown in red indicate parts changed from the original SNIPER(ER)-87.

Figure 2: Novel SNIPER(ER)s with potent protein knockdown activity. MCF-7 cells were treated with the indicated concentrations of SNIPER(ER)s for the indicated periods. Whole-cell lysates were analyzed by western blotting with the indicated antibodies. Numbers below the ERα, cIAP1 or XIAP panels represent ERα/actin, cIAP1/actin or XIAP/actin ratios, normalized by designating the expression from the vehicle control condition as 100%. Data in the bar graph are the mean ± S.D. of three independent experiments.

Figure 3: Protein knockdown activities of SNIPER(ER)s in T47D and ZR-75-1 breast tumor cells. (a) Protein levels of IAPs and ERα in human ERα-positive breast tumor cells. (b) Knockdown activities of SNIPER(ER)s in T47D and ZR-75-1 cells. Cells were treated with the indicated concentrations of SNIPER(ER)s for the indicated periods. Whole-cell lysates were analyzed by western blotting with the indicated antibodies. Numbers below the ERα, cIAP1 or XIAP panels represent ERα/actin, cIAP1/actin or XIAP/actin ratios, normalized by designating the expression from the vehicle control condition as 100%. Data in the bar graph are the mean ± S.D. of three independent experiments.

Figure 4: Role of XIAP in ERα degradation by SNIPER(ER)s. (a) SNIPER(ER)s preferentially recruit XIAP to ERα. MCF-7 or T47D cells were treated with 10 nM SNIPER(ER)s in the presence of 10 μM MG132 for 3 h. Immunoprecipitates of anti-ERα (IP) and whole-cell lysates (total lysates) were analyzed by western blotting. (b) Depletion of XIAP suppresses the SNIPER(ER)-induced degradation of ERα. MCF-7 and T47D cells were transfected with the indicated siRNA for 42 h and treated with 10 nM SNIPER(ER)s for 3 h. Whole-cell lysates were analyzed by western blotting with the indicated antibodies. Numbers below the ERα panel represent the ERα/actin ratio, normalized by designating the expression from the vehicle control condition as 100%. A mixture of three different siRNAs against each target was used to suppress expression.

Figure 5: Induction of apoptosis by SNIPER(ER)s in MCF7 breast tumor cells. (a)
Anti-proliferative effects of ERα-positive human breast tumor cells by SNIPER(ER)s. Cells were treated with the indicated concentrations of SNIPER(ER)s or fulvestrant for 72 h; remaining living cells were stained with crystal violet. (b - e) SNIPER(ER)s induce apoptosis in MCF-7 cells. MCF-7 or T47D cells were treated with 100 nM SNIPER(ER)s or fulvestrant for 48 h. (b) Phase-contrast images were obtained. Scale bars = 50 µm. (c) Cell cycle distribution was quantified by MultiCycle software. (d) Cell death was determined by Annexin V and PI staining. (e) Activation of caspases was analyzed by western blotting.

**Figure 6: Depletion of IAPs induces apoptosis in MCF-7 but not T47D cells.** MCF-7 or T47D cells were transfected with the indicated siRNA for 72 h. (a) Cell proliferation was evaluated by cell viability assay. (b) Activation of caspases was analyzed by western blotting. A mixture of three different siRNAs against each target was used to suppress expression.

**Figure 7: In vivo protein knockdown by SNIPER(ER)s in mice.** (a) In vivo protein knockdown in ovarian tissue. Female BALB/c mice were injected with vehicle or 30 mg/kg SNIPER(ER)s. After 24 or 48 h, the mice were sacrificed, and their ovaries were collected and analyzed by western blotting with the indicated antibodies. Numbers below the ERα panel represent the ERα/actin ratio, normalized by designating the expression from the vehicle control condition as 100% (average of each group). (b, c) MCF-7 human breast tumor cells were inoculated into mammary fat pads of 6-week-old female BALB/c nude mice. The tumor-bearing mice were intraperitoneally injected with SNIPER(ER)s. After 24 or 48 h, mice were sacrificed and ERα protein levels in tumor xenografts were analyzed by western blotting. Bar graphs represent the mean ± S.D. of each group. * p < 0.05 in two-sided Student’s t test compared with vehicle control. # p < 0.05 in two-sided Student’s t test compared with SNIPER(ER)-87.

**Figure 8: Antitumor activity of SNIPER(ER)s.** Growth inhibition by SNIPER(ER)s of MCF-7 orthotopic breast tumor xenografts in nude mice. (a) Representative tumors are shown. Scale bar = 10 mm. The tumor volume (b) or weight (c) represents the mean ± S.D. of each group (mice n = 7 each; tumor n = 14 each; * p < 0.05 in two-sided Student’s t test compared with vehicle, # p < 0.05 in two-sided Student’s t test compared with SNIPER(ER)-87). Mice were administered vehicle or SNIPER(ER)s (30 mg/kg) intraperitoneally, every 24 h. (d) Treatment with SNIPER(ER)s did not induce significant body weight loss in mice after 16 days.
Figure 1

SNIPER(ER)-104 (9)
SNIPER(ER)-87
Original Structure
IAP ligand module
SNIPER(ER)-105 (4)
SNIPER(ER)-110 (m-) (31a)
-113 (p-) (31c)
-119 (o-) (31d)
SNIPER(ER)-118 (m-) (55a)
-121 (p-) (55b)
SNIPER(ER)-134 (m-) (31b)
(A stereo-isomer of -110)
SNIPER(ER)-136 (40)
SNIPER(ER)-130 (69)
SNIPER(ER)-131 (71)
SNIPER(ER)-126 (50)
Figure 3

4h

4h

75
50
50
37

Mr (K)
(nM)

ERα
β-actin

100 29 29 27 40

ERα/actin

SN-87
56 24

SN-105 SN-110

10 1000 10 100 10 100

SN-126

10 100

cIAP1

XIAP

100 75 75 95 68 25 18 70

cIAP1/actin

XIAP/actin

100 37 39 31 75 65 80 97

100 49 36 21 40 22 36 21

100 91 80 65 97 81 87 92

T47D

ZR-75-1

MCF7
Figure 4

a

b
Figure 5

**a**

Graph showing cell number (% of control) against concentration (μM) for MCF7, T47D, and MDA-MB-231.

**b**

Images of DMSO, SN(ER)-87, SN(ER)-105, SN(ER)-110, SN(ER)-126, and Fulvestrant treated MCF7 and T47D cells.

**c**

Detailed images of propidium iodide (PI) and FITC Annexin V stained MCF7 and T47D treated with DMSO, SN(ER)-87, SN(ER)-105, SN(ER)-110, SN(ER)-126, and Fulvestrant.

**d**

Heat maps showing the ratio of propidium iodide (PI) to FITC Annexin V stained MCF7 treated with DMSO, SN(ER)-87, SN(ER)-105, SN(ER)-110, SN(ER)-126, and Fulvestrant.

**e**

Western blot images of PARP, Lamin B1, Caspase-7 (low expo.), Caspase-7 (high expo.), Caspase-6 (low expo.), Caspase-6 (high expo.), FLIP, ER, XIAP, cIAP1, and β-actin for MCF7 treated with DMSO, SN(ER)-87, SN(ER)-105, SN(ER)-110, SN(ER)-126, and Fulvestrant.
Figure 6

a  
MCF7  
T47D  

b  
MCF7  
T47D  

si-control (pmol)  
si-ERα (pmol)  
si-cIAP1 (pmol)  
si-XIAP (pmol)  

Cell Number (% of control)  

Lamin B1  
PARP  
Caspase-7  
ERα  
cIAP1  
XIAP  
β-actin  

Molecular weight (KDa)  

si-control (pmol)  
si-ERα (pmol)  
si-cIAP1 (pmol)  
si-XIAP (pmol)  

Cell Number (% of control)  

Lamin B1  
PARP  
Caspase-7  
ERα  
cIAP1  
XIAP  
β-actin  

Molecular weight (KDa)  

si-control (pmol)  
si-ERα (pmol)  
si-cIAP1 (pmol)  
si-XIAP (pmol)  

Cell Number (% of control)  

Lamin B1  
PARP  
Caspase-7  
ERα  
cIAP1  
XIAP  
β-actin  

Molecular weight (KDa)  

si-control (pmol)  
si-ERα (pmol)  
si-cIAP1 (pmol)  
si-XIAP (pmol)  

Cell Number (% of control)  

Lamin B1  
PARP  
Caspase-7  
ERα  
cIAP1  
XIAP  
β-actin  

Molecular weight (KDa)
### Table 1: ERα and β-actin expression levels in tumor tissues

| Tumor No. | Vehicle | SN-87 (30mg/kg) | SN-110 (30mg/kg) | SN-126 (30mg/kg) | Mr (K) |
|-----------|---------|-----------------|-----------------|-----------------|-------|
| 1         | 100     | 120             | 120             | 120             | 50    |
| 2         | 100     | 120             | 120             | 120             | 50    |
| 3         | 100     | 120             | 120             | 120             | 50    |
| 4         | 100     | 120             | 120             | 120             | 50    |
| 5         | 100     | 120             | 120             | 120             | 50    |
| 6         | 100     | 120             | 120             | 120             | 50    |
| 7         | 100     | 120             | 120             | 120             | 50    |
| 8         | 100     | 120             | 120             | 120             | 50    |
| 9         | 100     | 120             | 120             | 120             | 50    |
| 10        | 100     | 120             | 120             | 120             | 50    |
| 11        | 100     | 120             | 120             | 120             | 50    |
| 12        | 100     | 120             | 120             | 120             | 50    |

### Figure 7

#### a

- **Figure 7a:** Graph showing % of control (ERα/β-actin) for different treatments.
- **Figure 7b:** Graph showing % of control (cIAP1/β-actin) for different treatments.
- **Figure 7c:** Graph showing % of control (XIAP/β-actin) for different treatments.

#### b

- **Figure 7b:** Graph showing % of control (ERα/β-actin) for different treatments.
- **Figure 7c:** Graph showing % of control (cIAP1/β-actin) for different treatments.
- **Figure 7d:** Graph showing % of control (XIAP/β-actin) for different treatments.

#### c

- **Figure 7c:** Graph showing % of control (ERα/β-actin) for different treatments.
- **Figure 7d:** Graph showing % of control (cIAP1/β-actin) for different treatments.
- **Figure 7e:** Graph showing % of control (XIAP/β-actin) for different treatments.
Figure 8

a

Vehicle SN(ER)-87 SN(ER)-110

Tumor volume (mm³)

Days of treatment 2 4 6 8 10 12 14 16

* * * * * *

b

Tumor volume (mm³)

Days of treatment

c

Tumor weight (mg)

Vehicle SN(ER)-87 SN(ER)-110

* n.s.

n.s.

Vehicle SN(ER)-87 SN(ER)-110

Body weight (g)

Days of treatment

0 2 4 6 8 10 12 14 16

* * * * * *
Derivatization of inhibitor of apoptosis protein (IAP) ligands yields improved inducers of estrogen receptor α degradation
Nobumichi Ohoka, Yoko Morita, Katsunori Nagai, Kenichiro Shimokawa, Osamu Shibata, Takayuki Hattori, Tomoya Sameshima, Osamu Sano, Ryokichi Koyama, Yasuhiro Imaeda, Hiroshi Nara, Nobuo Cho and Mikihiro Naito

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