Cancer Science
The official journal of the Japanese Cancer Association

Development of hybrid small molecules that induce degradation of estrogen receptor-alpha and necrotic cell death in breast cancer cells

Keiichiro Okuhira,[1] Yosuke Demizu,[2] Takayuki Hattori,[1] Nobumichi Ohoka,[1] Norihito Shibata,[1] Tomoko Nishimaki-Mogami,[1] Haruhiro Okuda,[2] Masaaki Kurihara[2] and Mikihiko Naito[1,3]

Divisions of [1]Biochemistry and Molecular Biology, [2]Organic Chemistry, National Institute of Health Sciences, Tokyo, Japan

(Received June 3, 2013/Revised August 14, 2013/Accepted August 19, 2013/Accepted manuscript online August 30, 2013/Article first published online October 11, 2013)

Manipulation of protein stability with small molecules has a great potential for both basic research and clinical therapy. Recently, we have developed a series of hybrid small molecules named SNIPER (Specific and Non-genetic IAP-dependent Protein ERaser) that induces degradation of target proteins via ubiquitin-proteasome system. Here we report the activities of SNIPER(ER) that targets estrogen receptor alpha (ERα) for degradation. SNIPER(ER) induced degradation of ERα and inhibited estrogen-dependent expression of pS2 gene in an estrogen-dependent breast cancer cell line MCF-7. A proteasome inhibitor MG132 and siRNA-mediated downregulation of cIAP1 abrogated the SNIPER (ER)-induced ERα degradation, suggesting that the ERα is degraded by proteasome subsequent to cIAP1-mediated ubiquitylation. Intriguingly, after the ERα degradation, the SNIPER(ER)-treated MCF-7 cells undergo rapid cell death. Detailed analysis indicated that SNIPER(ER) caused necrotic cell death accompanied by a release of HMGB1, a marker of necrosis, from the cells. Following the ERα degradation, reactive oxygen species (ROS) was produced in the SNIPER(ER)-treated MCF-7 cells, and an antioxidant N-acetylcysteine inhibited the necrotic cell death. These results indicate that SNIPER(ER) induces ERα degradation, ROS production and necrotic cell death, implying a therapeutic potential of SNIPER(ER) as a lead for the treatment of ERα-positive breast cancers. (*Cancer Sci* 2013; 104: 1492–1498)

Breast cancer is the leading cause of cancer death in women.[1] Approximately, 75% of breast cancers express estrogen receptor α (ERα) and exhibit estrogen-dependent proliferation.[2] Estrogen receptor α is a member of the nuclear receptor family. The binding of estrogen to the ERα triggers its conformational change and the receptor dimerization, and facilitates binding of the receptor complex with co-regulators to the promoter of target genes to activate transcription,[3] resulting in the proliferation and growth of cancer cells. Tamoxifen, a selective ER modulator, is the most widely used anti-cancer drug in hormonal treatment of primary breast cancers. It competitively inhibits the binding of estrogen to ERα, and the conformational change of ERα induced by tamoxifen favors the recruitment of co-repressors that inhibit transcriptional activity,[4] and thus it causes cell cycle arrest and inhibition of cell growth.

Whereas tamoxifen antagonizes estrogen in breast cancers, it also shows a partial agonistic activity in different tissues and cellular context, which sometimes causes a detrimental effect. In particular, tamoxifen activates ER-regulated gene response in uterus, which is correlated with an increased incidence of the endometrial cancer.[5] An elevated level of co-activators in HepG2 cells has been reported to enhance the estrogenic properties of tamoxifen.[6] Furthermore, up to 40% of early-stage breast cancer patients who receive tamoxifen eventually develop resistance and relapse with a more aggressive cancer,[7] which is associated with the activation of "non-genetic" signal transduction pathway elicited by the tamoxifen binding to cytosolic or membrane-localized ERα[8] and ERα variants.[9] Thus, tamoxifen has a double face in regulating estrogen signaling, which hampers the safety and utility of this drug in the treatment of breast cancers.

An alternative strategy to kill the estrogen signaling is to downregulate ERα protein. We have developed a protein knockdown system to induce degradation of target proteins via the ubiquitin-proteasome system (UPS) in cells.[10–15] The molecules for protein knockdown, which we named SNIPER (Specific and Non-genetic IAP-dependent Protein ERaser), are composed of two distinct molecule that are chemically linked as a single molecule; one is a (−)-*N*-[(2*S*, 3*R*)-3-amino-2-hydroxy-4-phenyl-butyryl]-$_L$-leucine (bestatin, BS) and the other is a ligand for a target protein. Our earlier work demonstrated that the methyl ester of bestatin (MeBS) directly interacts with BIR3 domain of cellular inhibitor of apoptosis protein 1 (cIAP1) and induces auto-ubiquitylation of cIAP1 depending on its RING domain, resulting in the proteasomal degradation of cIAP1.[16] Structure-activity relationship study indicated that the MeBS analogs degrade cIAP1 even though the methyl group is substituted to other residues.[16] Based on these observations, SNIPERs consisting of MeBS (a ligand for cIAP1) and another ligand for a target protein were developed to cross-link cIAP1 and the target protein in the cells. Thus, SNIPERs are expected to induce cIAP1-mediated ubiquitylation and proteasomal degradation of the target protein, although the precise protein-knockdown mechanism of the individual SNIPERs must be clarified. Recently, we designed and synthesized novel SNIPERs targeting ER (SNIPER[ER]) by using 4-hydroxy tamoxifen (4-OHT) as an ERα ligand (Fig. 1a). In this paper, we reported the molecular mechanism by which SNIPER(ER) degrades the ERα protein. We also showed that SNIPER(ER) induces ROS production and necrotic cell death in the ERα-expressing breast cancer cells. We propose targeted destruction of oncogenic proteins could be a novel strategy to treat cancers.

Materials and Methods

Reagents. SNIPER(ER)s were synthesized as described previously.[15] (−)-*N*-[(2*S*, 3*R*)-3-amino-2-hydroxy-4-phenyl-butyryl]-$_L$-leucine methyl ester (MeBS) was kindly provided by Nippon Kayaku, (Tokyo, Japan). The following reagents were purchased from indicated supplier: anti-ERα mouse

[3]To whom correspondence should be addressed.
E-mail: miki-naito@nihs.go.jp

doi: 10.1111/cas.12272

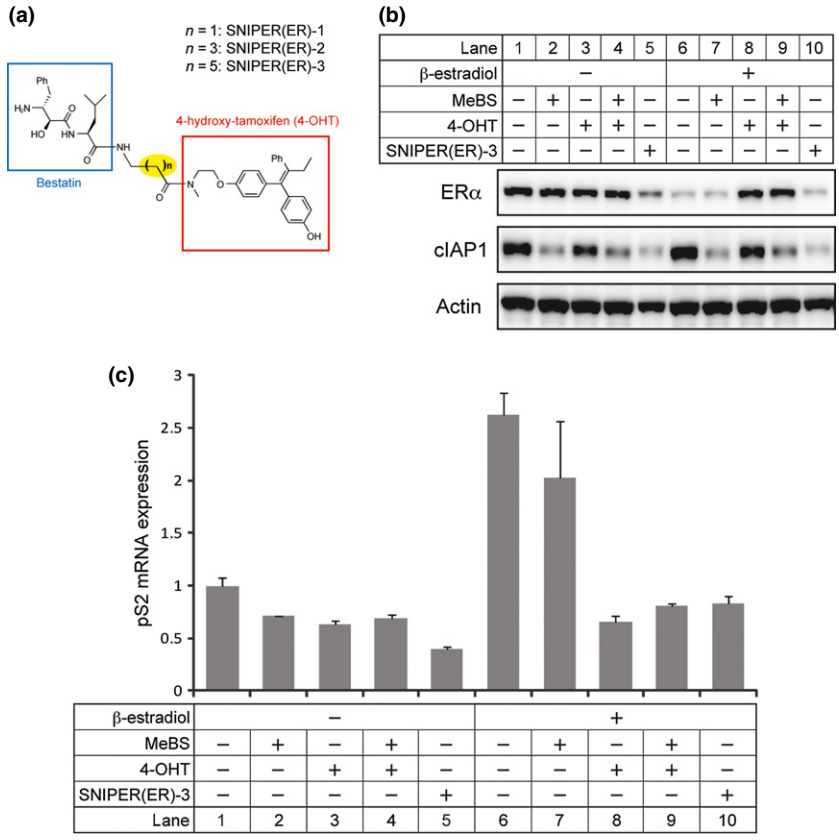

Fig. 1. SNIPER(ER) reduced estrogen receptor α (ERα) protein level and inhibited the upregulation of ERα-regulated gene pS2 expression by β-estradiol. (a) Structure of SNIPER(ER)s. (b) MCF-7 cells precultured in the media containing estrogen-depleted serum were treated with 10 μM of compounds (MeBS, 4-OHT, SNIPER(ER)-3) in the absence or presence of 10 nM of β-estradiol for 9 h. Shown are immunoblots of cell lysates stained with indicated antibodies. (c) pS2 mRNA level was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and normalized with 18S rRNA. Values are expressed as the fold change relative to control.

monoclonal antibody, anti-actin mouse monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA); anti-ERα rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA); anti-β-actin mouse monoclonal antibody, N-acetylcysteine (Sigma-Aldrich, St Louis, MO, USA); anti-human cIAP1 goat polyclonal antibody (R&D Systems, Minneapolis, MN, USA); anti-HMGB1 rabbit monoclonal antibody (Abcam, Cambridge, UK); hydrogen peroxide (H_2O_2) (Wako Pure Chemical Industries, Osaka, Japan); staurosporine (Enzo Life Sciences, Farmingdale, NY, USA); MG132 (Peptide Institute, Osaka, Japan); Lipofectamine RNAi MAX transfection reagent, Stealth Select RNAi (Life Technologies, Carlsbad, CA, USA). The target sequences for cIAP1 RNAi were as follows; cIAP1-#1 (5′-GCTGTAGCTTTATTCAGAATCTGGT-3′); cIAP1-#2 (5′-GGAAATGCTGCGGCCAACATCTTCA-3′).

Cell culture, transfection and treatment with compounds. Human breast cancer MCF-7 and T47D were maintained in RPMI 1640 medium containing 10% FBS and 60 μg/mL of kanamycin. HeLa and fibrosarcoma U2OS cells were maintained in DMEM medium containing 10% FBS and 60 μg/mL of kanamycin. siRNA transfections were carried out by reverse transfection method with Lipofectamine RNAi MAX according to the manufacturer's instructions. In some experiments, MCF-7 cells were precultured in phenol-red free RPMI 1640 medium containing 4% charcoal/dextran-treated FBS (HyClone Laboratories, Logan, UT, USA) and kanamycin for over 72 h and then in the medium containing 0.2% charcoal/dextran-treated FBS and kanamycin for 24 h. Cells were then treated with indicated concentration of compounds including SNIPER(ER)s for indicated times.

Western blot analysis. Cells were lysed in lysis buffer (1% SDS, 0.1 M Tris-HCl, pH 7.0, 10% glycerol) and boiled for 10 min. Protein concentration was determined by BCA method (Thermo Scientific, Rockford, IL, USA). The equal amount of protein cell lysate or the equal volumes of cell culture medium were separated by SDS-PAGE, transferred to Hybond-P (GE Healthcare, Buckinghamshire, UK) membrane and Western blotted using indicated antibodies. Protein signals were detected using SuperSignal West Dura or West Femto Substrate (Thermo Scientific).

RNA extraction and quantitative real time PCR. Total RNA was isolated using the RNeasy Mini kit according to manufacturer's instructions (Qiagen, Valencia, CA, USA). Purified RNA was reverse-transcribed and amplified by polymerase chain reaction with one-step RT-PCR Master Mix Reagents kit (Applied Biosystems, Branchburg, NJ, USA) in an ABI PRISM 7000 Sequence Detection system. The pS2 primers and probe used were; forward primer, 5′-CGTGAAAGACA GAATTGTGGTTTT-3′; reverse primer, 5′-CGTCGAAACAG CAGCCCTTA-3′; probe, 5′-TGTCACGCCCTCCCAGTGTG CA-3′. The data were normalized for 18S rRNA levels and were presented as fold change against DMSO-treated control cells.

Cell viability assay. Cell viability was evaluated by crystal violet staining or WST-8 assay (Cell Counting Kit-8; Dojindo, Kumamoto, Japan). For crystal violet staining, cells treated with compounds were stained with 0.1% crystal violet (Wako) in 1% ethanol for 15 min at room temperature. The cells were rinsed thoroughly with distilled water, and then lysed in 1% SDS. The absorbance at 600 nm of cell lysate was measured using EnVision Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA). For WST-8 assay, cells treated with compounds were incubated with WST-8 for 1 h in CO_2 incubator, and the absorbance at 450 nm was measured.

Propidium iodide and Hoechst staining. After the treatment with compounds, cells were stained with 2 μg/mL of propidium iodide (PI) and 2 μg/mL of Hoechst 33342 for 10 min. Fluorescence images were obtained by BZ-9000 (Keyence, Osaka, Japan).

Measurement of ROS. Intracellular ROS were detected using Cell ROX Green Reagent (Life Technologies). Cells were

incubated simultaneously with 30 μM of compounds and 5 μM of Cell ROX Reagent in phenol-red free culture medium for 5 h. Fluorescence images were obtained by BZ-9000 and the mean fluorescence intensity (green) was calculated using the Keyence BZ-9000 Analysis software.

Results

Degradation of ERα protein by SNIPER(ER). Upon estrogen binding, ERα translocates to nuclei and activates gene expression such as pS2, and then it is degraded via the UPS.[17] To discriminate the SNIPER-induced ERα degradation from the physiological degradation coupled with estrogen binding, MCF-7 cells were precultured in the media containing estrogen-depleted serum for over 4 days. Then, cells were treated with SNIPER(ER)-3 (Fig. 1a, $n = 5$) in the absence or presence of β-estradiol as an estrogen, and the ERα protein level was examined by western blot analysis (Fig. 1b). In the absence of β-estradiol, SNIPER(ER)-3 reduced the level of ERα protein (lane 5), whereas methyl bestatin (MeBS) (lane 2), 4-OHT (lane 3) or a combination of MeBS and 4-OHT (lane 4) did not affect the ERα level. This result indicates that SNIPER(ER)-3 in which 4-OHT and MeBS was conjugated as a single molecule shows an activity to reduce ERα protein level. SNIPER(ER)-3 also reduced the level of cIAP1 as did MeBS, indicating that SNIPER(ER)-3 simultaneously activates auto-ubiquitylation and proteasomal degradation of cIAP1 as observed with another SNIPER targeting CRABP-II protein.[11]

Treatment of the MCF-7 cells with β-estradiol reduced the ERα protein (lane 6), which was suppressed by 4-OHT (lane 8), suggesting that the ERα reduction by β-estradiol is coupled with ERα activation.[17] SNIPER(ER)-3 also reduced the ERα protein level in the presence of β-estradiol (lane 10). To examine if SNIPER(ER)-3 has an estrogenic activity, we measured the expression of ERα-regulated gene pS2 by quantitative real-time PCR. As shown in Figure 1(c), β-estradiol, but not SNIPER(ER)-3, induced the expression of pS2 mRNA. SNIPER (ER)-3 blocked the upregulation of pS2 induced by β-estradiol, as did 4-OHT (lane 10). These results clearly indicate that SNIPER(ER)-3 reduced the ERα protein level independent on the activation of ERα.

We also examined the effects of SNIPER(ER) on the ERα protein level under normal culture condition, that is, in the media supplemented with 10% FBS. Since FBS contains a significant concentration of estrogens, 4-OHT increased the ERα protein level (Fig. 2a, lanes 3 and 10) as observed in the β-estradiol-treated MCF-7 cells in the estrogen-depleted culture (Fig. 1b, lane 8). When compared with the 4-OHT (lane 3) or the combination of 4-OHT and MeBS (lane 4), SNIPER (ER)s with different linker length reduced the ERα protein levels (lanes 5–7). Although there were no apparent difference in the activities of these three SNIPER(ER)s at 6 h, SNIPER (ER)-3 appeared to have a most potent effect on the ERα level

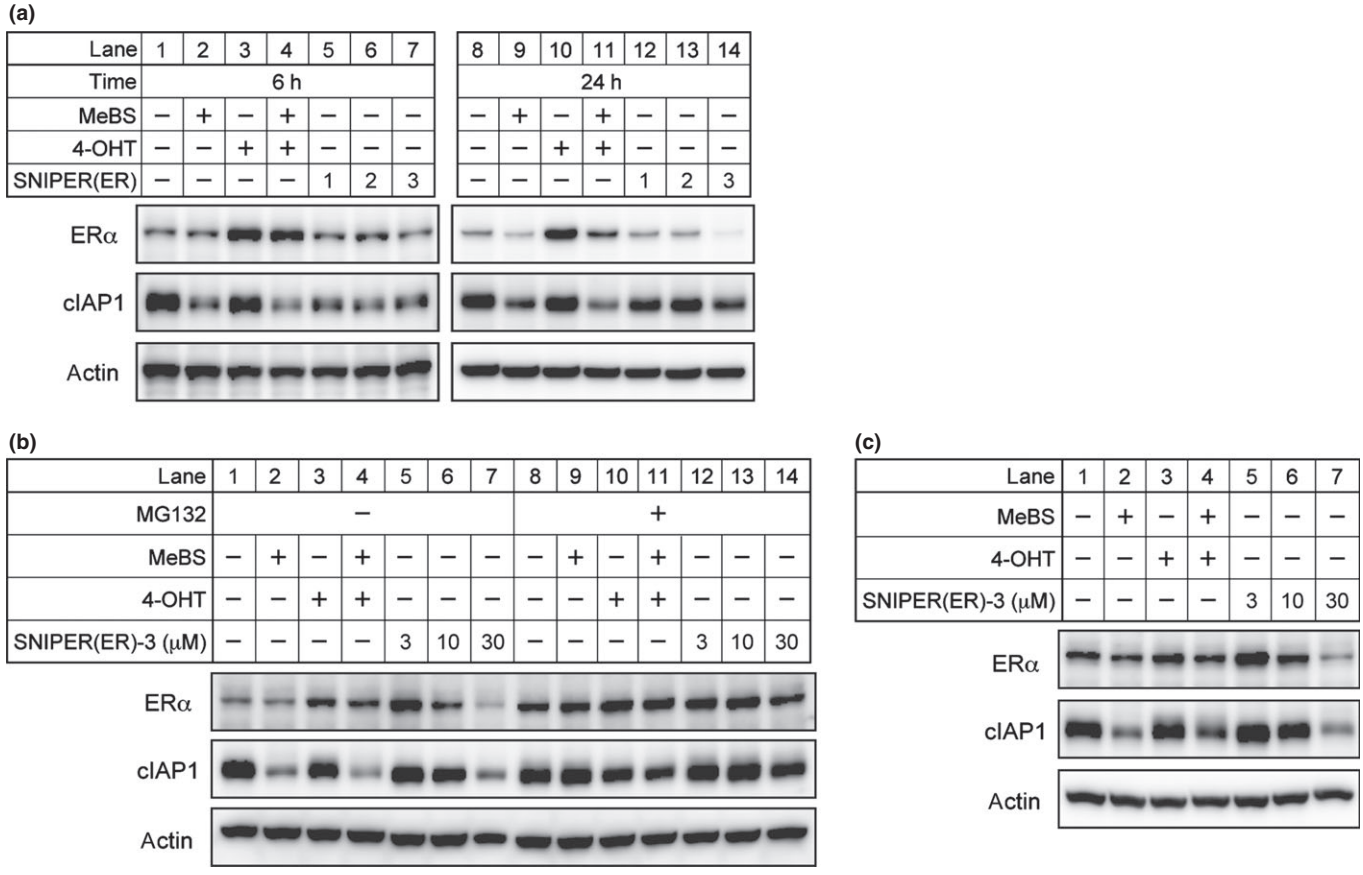

Fig. 2. Degradation of estrogen receptor α (ERα) protein by SNIPER(ER). (a) MCF-7 cells cultured in the media containing 10% fetal bovine serum (FBS) were treated with 10 μM of compounds (MeBS, 4-OHT, SNIPER(ER)-1, -2, -3) for 6 h or 24 h. Shown are immunoblots of cell lysates stained with indicated antibodies. (b) Proteasome inhibitor MG132 inhibited the ERα and cIAP1 degradation. MCF-7 cells were treated with compounds for 6 h in the presence or absence of 10 μM MG132. (c) SNIPER(ER)-3 degraded ERα in T47D breast cancer cells. T47D cells were treated with compounds for 12 h.

doi: 10.1111/cas.12272

at 24 h. Therefore, we focused on the activity of SNIPER (ER)-3 in this study.

Figure 2(b) shows that SNIPER(ER)-3 at 3 μM rather increased the ERα protein level as did 4-OHT (lane 5). This might be due to an antagonistic activity of SNIPER(ER)-3 derived from the 4-OHT moiety of this compound. At 30 μM, SNIPER(ER)-3 clearly reduced ERα and cIAP1 (lane 7). The reduction of ERα and cIAP1 by SNIPER(ER)-3 was abrogated by a proteasome inhibitor, MG132 (lanes 12–14), indicating that SNIPER(ER)-3 induces the proteasomal degradation of ERα and cIAP1. The reduction of ERα protein by SNIPER (ER)-3 was similarly observed in another breast cancer cell line T47D expressing ERα protein (Fig. 2c).

We next examined whether cIAP1 is involved in the degradation of ERα induced by SNIPER(ER)-3. Silencing of cIAP1 expression by siRNAs (#1 and #2) significantly suppressed the SNIPER(ER)-3-induced ERα protein degradation (Fig. 3). This result suggests that cIAP1 is the primary ubiquitin ligase for ERα degradation when treated with SNIPER(ER)-3.

SNIPER(ER)-3 induces necrotic cell death in MCF-7 cells. Upon the treatment with higher concentration of SNIPER(ER)-3, we observed that MCF-7 cells underwent rapid cell death (Fig. 4a, b). Treatment with MeBS, 4-OHT, or the combination of them did not affect the cell viability (Fig. 4b). The cell death was confirmed by the inability to exclude PI (Fig. 4c). MCF-7 cells treated with SNIPER(ER)-3 and hydrogen peroxide were stained with PI, indicating a loss of plasma membrane integrity as a result of cell death. Interestingly, SNIPER(ER)-3 did not induce the cell death in U2OS or HeLa cells, both of which do not express the ERα protein (Fig. 4a,b), nor in MCF-7 cells treated with MG132 (Fig. 4a–c). These results strongly suggest that ERα degradation by SNIPER(ER)-3 triggers the rapid cell death in MCF-7 cells.

To further investigate the type of cell death induced by SNIPER(ER)-3, we examined the involvement of caspases in the cell death. However, a pan-caspase inhibitor zVAD-FMK did not inhibit the SNIPER(ER)-3-induced cell death (data not shown). Then, we examined the release of high mobility group box 1 (HMGB1) protein from SNIPER(ER)-3-treated cells. HMGB1 is a chromatin-associated protein that is released into the extracellular environment during cell necrosis, but not apoptosis.[18] SNIPER(ER)-3 induced the release of HMGB1 into the culture media, as did hydrogen peroxide, which was suppressed by MG132 (Fig. 4d). The HMGB1 was not released from the cells treated with MeBS, 4-OHT, the combination of them, or staurosporine, an apoptosis inducer (Fig. 4d). These results indicate that SNIPER(ER)-3 induces necrotic cell death in MCF-7 cells.

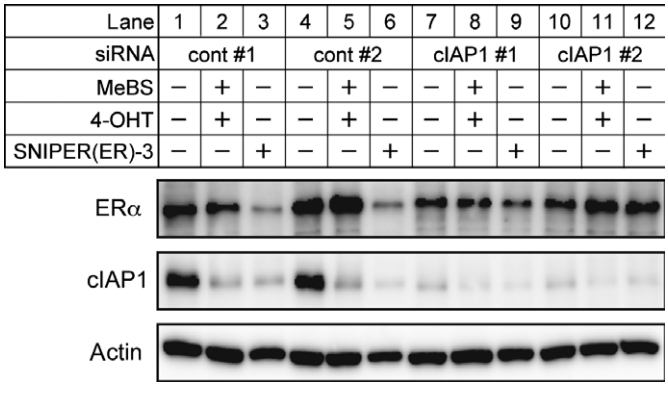

Fig. 3. Silencing of cIAP1 attenuates the SNIPER(ER)-dependent ERα protein degradation. In MCF-7 cells, endogenous cIAP1 were knocked down by two different siRNAs (#1 or #2) for 48 h. Then, cells were treated with 30 μM of compounds for 6 h. Shown are immunoblots of cell lysates stained with indicated antibodies.

Reactive oxygen species is involved in the SNIPER(ER)-3-induced necrotic cell death. Since ROS mediates necrotic cell death in a variety of cell system,[19] we evaluated the ROS production in MCF-7 cells (Fig. 5a,b). We found that SNIPER (ER)-3 strongly, and MeBS plus 4-OHT slightly, induced the ROS production in MCF-7 cells. Individual treatment with MeBS negligibly and 4-OHT slightly induced the ROS production (data not shown). The SNIPER(ER)-3-induced ROS production in MCF-7 cells was suppressed by MG132. In addition, the ROS production in U2OS cells by SNIPER(ER)-3 was not significantly different from the cells treated with MeBS plus 4-OHT. These results suggest that ROS is abundantly produced in the SNIPER(ER)-3-treated MCF-7 cells after the ERα protein degradation.

Finally, we examined whether the inhibition of ROS attenuates the SNIPER(ER)-3-induced necrotic cell death. Treatment with N-acetylcysteine (NAC), a ROS scavenger, inhibited the HMGB1 release induced by SNIPER(ER)-3 (Fig. 5c), and suppressed necrotic cell death of MCF-7 cells (Fig. 5d). N-acetylcysteine did not block the ERα degradation (Fig. 5c), confirming that ROS production is as an event downstream of ERα degradation. These results indicate that ROS is involved in the necrotic cell death induced by SNIPER(ER)-3.

Discussion

In this study, we demonstrated that SNIPER(ER) induces proteasomal degradation of ERα proteins without inducing ERα-mediated gene expression. The involvement of cIAP1 in the SNIPER(ER)-induced ERα degradation strongly suggests that the degradation depends on the cIAP1-mediated ubiquitylation of ERα. Thus, although ERα is physiologically degraded by proteasome after activation by estrogens, SNIPER(ER) induces ERα degradation in a different mechanism. It is likely that SNIPER(ER) crosslinks cIAP1 and ERα, and induces cIAP1-mediated ubiquitylation of ERα in the cells. The ternary complex composed of cIAP1, SNIPER(ER) and ERα could be formed in the cells at a higher concentration of SNIPER(ER), resulting in a degradation of ERα. At a lower concentration of SNIPER(ER), however, the ternary complex would not be formed. Since the affinity of MeBS to cIAP1 is a hundred times lower than that of 4-OHT to ERα, SNIPER (ER) preferentially binds to ERα compared to cIAP1 at a lower concentration. In line with this, SNIPER(ER) at a lower concentration rather increased ERα, but did not affect the cIAP1 level (Fig. 2b). Thus, SNIPER(ER) has a dual activity on ERα depending on its concentration; at lower concentration it inhibits ERα as an antagonist like 4-OHT, while at a higher concentration it induces ERα degradation as a SNIPER.

Intriguingly, SNIPER(ER) induced necrotic cell death in ERα-positive breast cancer MCF-7 cells, but not in ERα-negative U2OS and HeLa cells, nor in MCF-7 cells treated with proteasome inhibitor. Silencing of ERα by siRNA in combination with MeBS to reduce cIAP1 did not cause the necrotic cell death (data not shown), suggesting that the reduction of ERα and cIAP1 protein levels are not sufficient to induce necrotic cell death. As an additional mediator for the SNIPER (ER)-induced necrosis, ROS is produced in the SNIPER(ER)-treated cells. The ROS production occurs after ERα degradation, since the proteasome inhibitor abrogated both the ERα degradation (Fig. 2b) and ROS production (Fig. 5a,b), while NAC did not suppress ERα degradation (Fig. 5c). HMGB1 release from the cells and necrotic cell death were suppressed by both MG132 (Fig. 4a–d) and NAC (Fig. 5c,d). Taken together, we speculate that SNIPER(ER) induces necrotic cell

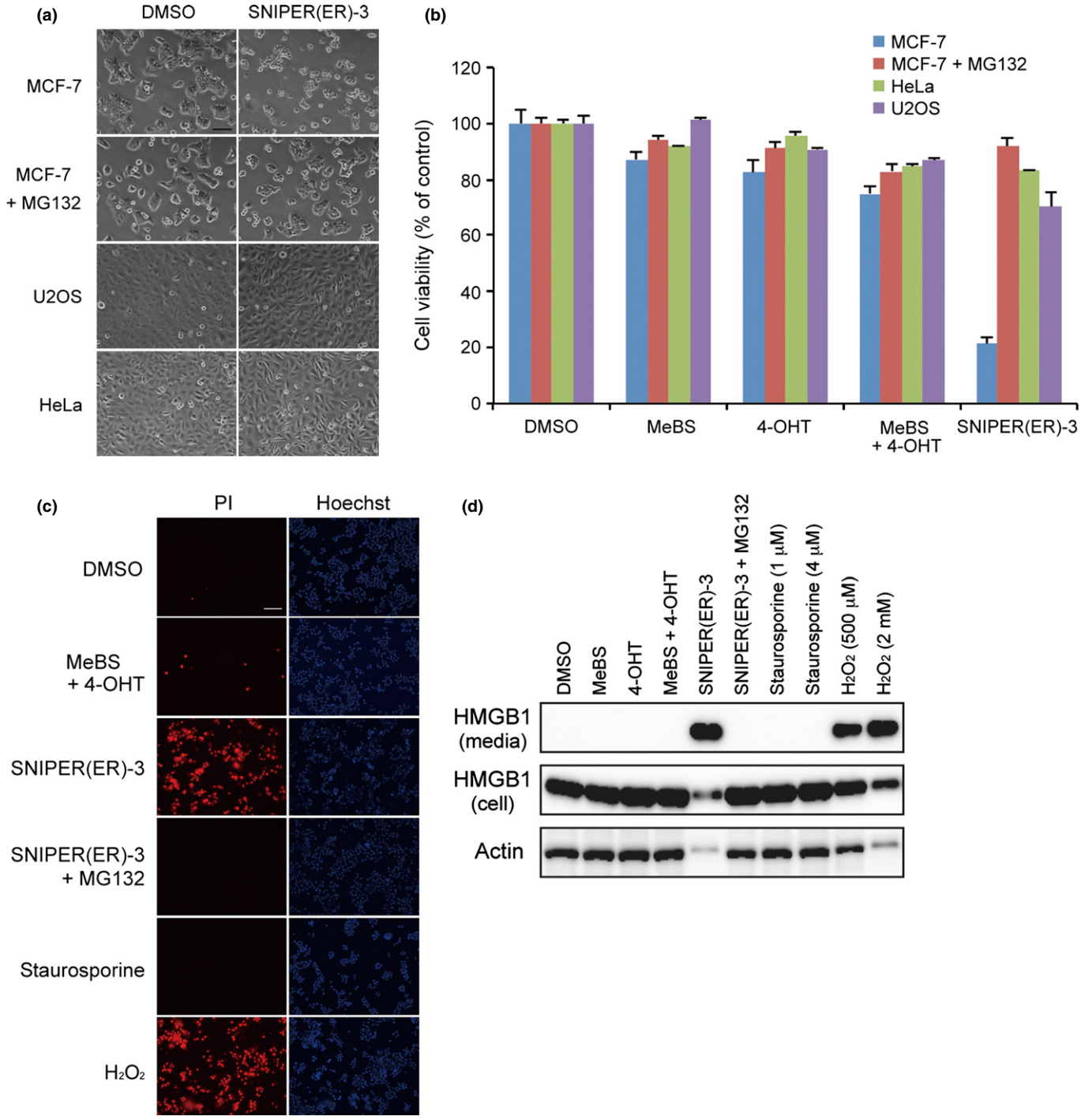

Fig. 4. SNIPER(ER) induced necrotic cell death in MCF-7 cells. (a) MCF-7, U2OS or HeLa cells were treated with 30 μM of SNIPER(ER)-3 for 6 h in the presence or absence of 10 μM MG132. Bar: 100 μm. (b) Cell viability was determined by crystal violet staining and calculated as values relative to control cells. Values are the means ± standard deviation (SD) of triplicate samples. (c) MCF-7 cells were treated with MeBS plus 4-OHT (30 μM), SNIPER(ER)-3 (30 μM), staurosporine (1 μM) or hydrogen peroxide (500 μM) for 6 h. Cells were stained with propidium iodide (PI) and Hoechst 33342, and analyzed by fluorescence microscopy. Bar: 100 μm. (d) SNIPER(ER)-3 induced HMGB1 release. MCF-7 cells were treated with MeBS (30 μM), 4-OHT (30 μM), MeBS plus 4-OHT (30 μM), SNIPER(ER)-3 (30 μM), staurosporine (1 μM or 4 μM), hydrogen peroxide (500 μM or 2 mM) for 9 h. Shown are immunoblots of cell lysates and culture media stained with indicated antibodies.

death through a sequential event as illustrated in Figure 6. Currently, the mechanism by which SNIPER(ER) induces ROS production after ERα degradation is obscure. However, previous reports pointed out the molecular interactions between ERα and cellular superoxide dismutase (SOD), which is an enzyme that scavenges ROS.[20] It is tempting to speculate that SNIPER (ER) might influence the stability and/or activity of SOD by destabilizing ERα, resulting in the ROS production. Further studies would be needed to clarify the mechanism of how SNIPER(ER) induces ROS production and necrotic cell death.

doi: 10.1111/cas.12272

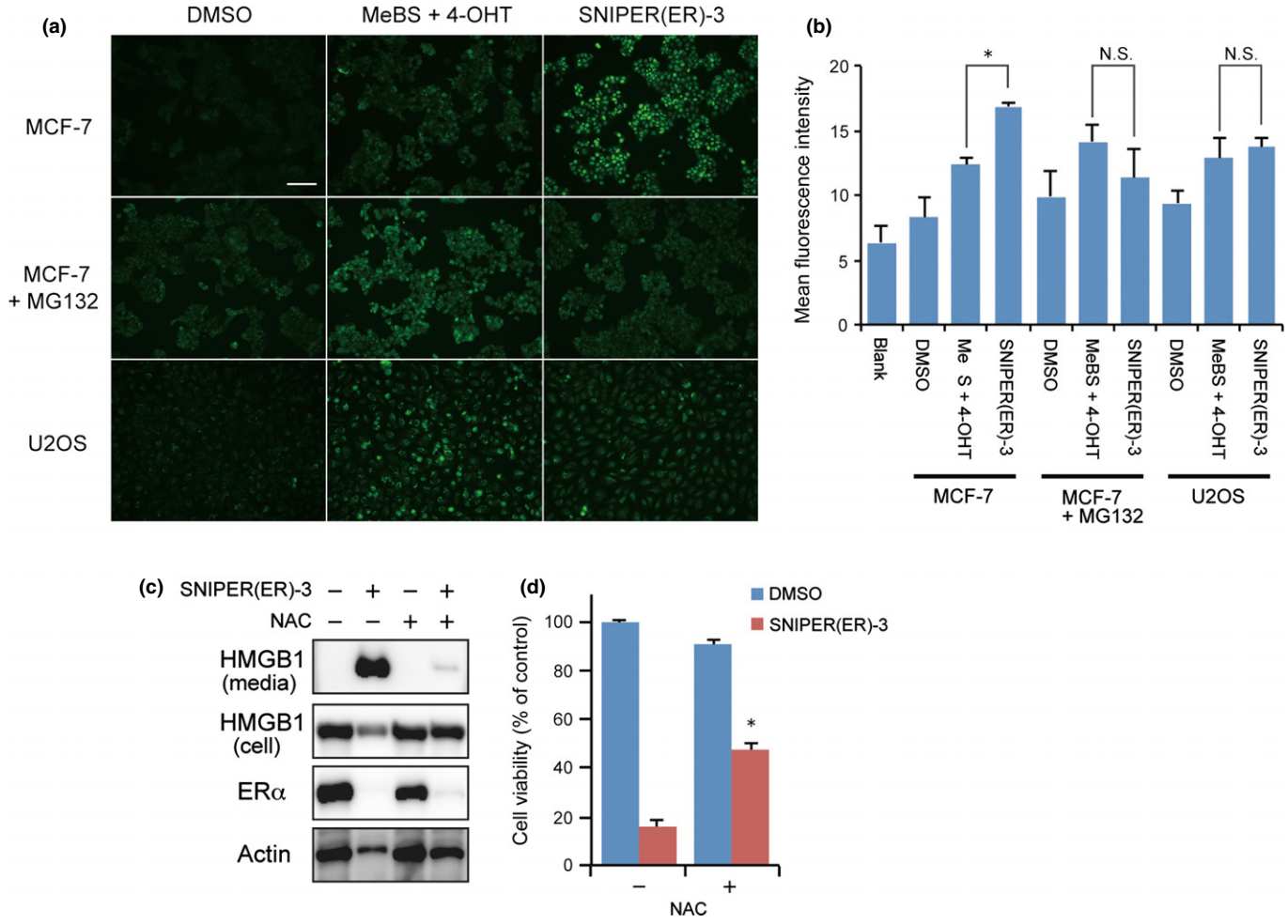

Fig. 5. Reactive oxygen species (ROS) is involved in the SNIPER(ER)-3-induced necrotic cell death. (a) SNIPER(ER)-3 increased ROS in MCF-7 cells, but not in U2OS cells, or MG132-treated MCF-7 cells. Cells were treated with 30 μM of MeBS plus 4-OHT or SNIPER(ER)-3 for 5 h. ROS was detected using Cell ROX Green reagent. Bar: 100 μm. (b) Mean fluorescence intensity of acquired images was calculated. Values are the means ± standard deviation (SD) of triplicate samples. An asterisk represents a statistically significant difference ($P < 0.001$, *t*-test). N.S., not significant. (c) ROS scavenger N-acetylcysteine (NAC) inhibited the HMGB1 release from SNIPER(ER)-3-treated cells. MCF-7 cells were treated with 10 μM of SNIPER(ER)-3 in the presence or absence of N-acetylcysteine (20 mM) for 37 h. Shown are immunoblots of cell lysates or media stained with indicated antibodies. (d) N-acetylcysteine inhibited the SNIPER(ER)-3-induced cell death. MCF-7 cells were treated with 10 μM of SNIPER(ER)-3 in the presence or absence of NAC (15 mM) for 37 h. Cell viability was measured by WST-8 assay. Values are the means ± SD of triplicate samples. An asterisk represents a statistically significant difference from the values at cells treated with SNIPER(ER) in the absence of NAC ($P < 0.001$, *t*-test).

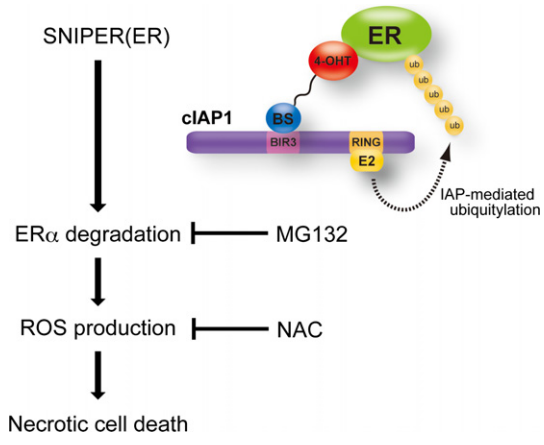

Fig. 6. Possible scheme of SNIPER(ER)-induced estrogen receptor α (ERα) protein degradation and necrotic cell death.

The modular structure of the SNIPER implies the potential application of this molecule to degrade other target proteins. Since the demonstration of the outstanding therapeutic effects of oncogenic kinase inhibitors, such as imatinib and crizotinib, many of the molecular target drugs currently under development intend to inhibit enzyme activity of the pathogenic proteins.[21] However, cancer cells often acquire resistance by mutating the protein at the active center of the kinases, which is a new problem recently emerging in clinic.[22,23] To overcome the drug resistance, novel kinase inhibitors are developed to inhibit the mutant kinases.[24,25] As an alternative strategy, SNIPER can be applicable to kill the kinase signaling by destruction of the proteins. Because most proteins have multiple domains that can be recognized by their ligands, several SNIPER molecules can be designed to target a single protein for degradation by using different ligands against different domains. Thus, the protein knockdown system with SNIPERs would expand a possibility to develop a variety of novel molecular target drugs.

Acknowledgments

We thank Nippon Kayaku, for kindly providing MeBS. This study was supported by Grants-in Aid for Scientific Research from the Japan Society for the Promotion of Science (to M.N. and K.O.), by Research Fund from Japan Health Sciences Foundation, by a research grant from the Astellas Foundation for Research on Metabolic Disorders, a grant from the Terumo Life Science Foundation (to M.N.) and a grant from Mochida Memorial Foundation for Medical and Pharmaceutical Research (to K.O.).

Disclosure Statement

M. Naito received a research fund from Takeda Pharmaceutical Company (Osaka, Japan).

Abbreviations

4-OHT	4-hydroxy tamoxifen
cIAP1	cellular inhibitor of apoptosis protein 1
ERα	estrogen receptor α
HMGB1	high mobility group box 1
MeBS	(−)-*N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl-butyryl]-$_L$-leucine methyl ester
ROS	reactive oxygen species
NAC	N-acetylcysteine
PI	propidium iodide
SNIPER	specific and non-genetic IAP-dependent protein eraser
UPS	ubiquitin-proteasome system

References

1 Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69–90.
2 Morales AR, Ganjei-Azar P, Gomez-Fernandez C, Nadji M. Immunohistochemistry of estrogen and progesterone receptors reconsidered. *Am J Clin Path* 2005; **123**: 21–7.
3 Hall JM, McDonnell DP. Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. *Mol Interv* 2005; **5**: 343–57.
4 Brzozowski AM, Pike AC, Dauter Z *et al.* Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 1997; **389**: 753–8.
5 Bernstein L, Deapen D, Cerhan JR *et al.* Tamoxifen therapy for breast cancer and endometrial cancer risk. *J Natl Cancer Inst* 1999; **91**: 1654–62.
6 Smith CL, Nawaz Z, O'Malley BW. Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. *Mol Endocrinol* 1997; **11**: 657–66.
7 Ring A, Dowsett M. Mechanisms of tamoxifen resistance. *Endocr Relat Cancer* 2004; **11**: 643–58.
8 Levin ER. Plasma membrane estrogen receptors. *Trends Endocrinol Metab* 2009; **20**: 477–82.
9 Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF. A variant of estrogen receptor-α, hER-α36: transduction of estrogen- and antiestrogen-dependent membrane-initiated mitogenic signaling. *Proc Natl Acad Sci USA* 2006; **103**: 9063–8.
10 Okuhira K, Ohoka N, Sai 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.
11 Itoh Y, Ishikawa M, Naito M, Hashimoto Y. Protein knockdown using methyl bestatin-ligand hybrid molecules: design and synthesis of inducers of ubiquitination-mediated degradation of cellular retinoic acid-binding proteins. *J Am Chem Soc* 2010; **132**: 5820–6.
12 Itoh Y, Ishikawa M, Kitaguchi R, Sato S, Naito M, Hashimoto Y. Development of target protein-selective degradation inducer for protein knockdown. *Bioorg Med Chem* 2011; **19**: 3229–41.
13 Itoh Y, Kitaguchi R, Ishikawa M, Naito M, Hashimoto Y. Design, synthesis and biological evaluation of nuclear receptor-degradation inducers. *Bioorg Med Chem* 2011; **19**: 6768–78.
14 Itoh Y, Ishikawa M, Kitaguchi R, Okuhira K, Naito M, Hashimoto Y. Double protein knockdown of cIAP1 and CRABP-II using a hybrid molecule consisting of ATRA and IAPs antagonist. *Bioorg Med Chem* 2012; **22**: 4453–7.
15 Demizu Y, Okuhira K, Motoi H *et al.* Design and synthesis of estrogen receptor degradation inducer based on a protein knockdown strategy. *Bioorg Med Chem* 2012; **22**: 1793–6.
16 Sekine K, Takubo K, Kikuchi R *et al.* Small molecules destabilize cIAP1 by activating auto-ubiquitylation. *J Biol Chem* 2008; **283**: 8961–8.
17 Valley CC, Metivier R, Solodin NM *et al.* Differential regulation of estrogen-inducible proteolysis and transcription by the estrogen receptor alpha N terminus. *Mol Cell Biol* 2005; **25**: 5417–28.
18 Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002; **418**: 191–5.
19 Zong WX, Thompson CB. Necrotic death as a cell fate. *Genes Dev* 2006; **20**: 1–15.
20 Rao AK, Ziegler YS, McLeod IX, Yates JR, Nardulli AM. Effects of Cu/Zn superoxide dismutase on estrogen responsiveness and oxidative stress in human breast cancer cells. *Mol Endocrinol* 2008; **22**: 1113–24.
21 Drenberg CD, Baker SD, Sparreboom A. Integrating clinical pharmacology concepts in individualized therapy with tyrosine kinase inhibitors. *Clin Pharmacol Ther* 2013; **93**: 215–9.
22 Choi YL, Soda M, Yamashita Y *et al.* EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. *N Engl J Med* 2010; **363**: 1734–9.
23 Katayama R, Shaw AT, Khan TM *et al.* Mechanisms of acquired crizotinib resistance in ALK-rearranged lung Cancers. *Sci Transl Med* 2012; **4**: 120ra17.
24 O'Hare T, Walters DK, Stoffregen EP *et al.* In vitro activity of Bcr-Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants. *Cancer Res* 2005; **65**: 4500–5.
25 Sakamoto H, Tsukaguchi T, Hiroshima S *et al.* CH5424802, a selective ALK inhibitor capable of blocking the resistant gatekeeper mutant. *Cancer Cell* 2011; **19**: 679–90.

doi: 10.1111/cas.12272