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Protein degradation for drug discovery

SNIPERs—Hijacking IAP activity to induce protein degradation

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Abstract

The induction of protein degradation by chimeric small molecules represented by proteolysis-targeting chimeras (PROTACs) is an emerging approach for novel drug development. We have developed a series of chimeric molecules termed specific and non-genetic inhibitor of apoptosis protein (IAP)-dependent protein erasers (SNIPERs) that recruit IAP ubiquitin ligases to effect targeted degradation. Unlike the chimeric molecules that recruit von Hippel–Lindau and cereblon ubiquitin ligases, SNIPERs induce simultaneous degradation of IAPs such as cIAP1 and XIAP along with the target proteins. Because cancer cells often overexpress IAPs—a mechanism involved in the resistance to cancer therapy—SNIPERs could be used to kill cancer cells efficiently.

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Introduction

Targeted protein degradation using chimeric molecules such as SNIPERs and PROTACs is a novel technology employed to manipulate protein stability [1–3]. Degradation is achieved through the ubiquitylation of target proteins by hijacking the activity of ubiquitin ligase. Proteins that are marked with poly-ubiquitin chains, such as K48-linked poly-ubiquitin, are then subjected to proteasomal degradation [4]. Of over 600 E3 ubiquitin ligases present in cells, only a limited number—including inhibitor of apoptosis proteins (IAPs), von Hippel-Lindau tumor suppressor (VHL) and cereblon (CRBN)—have been shown to be recruited to target proteins by chimeric molecules.

IAPs are a family of proteins that contain one to three baculoviral IAP repeat (BIR) domains. These proteins inhibit apoptosis in various cellular systems when they are overexpressed [5–8]. There are eight IAP proteins in humans (Fig. 1). Among them, five members (X-linked IAP [XIAP], cellular IAP1 [cIAP1], cIAP2, Livin and IAP-like protein 2 [ILP2]) contain a really interesting new gene (RING) finger domain which can interact with E2 ubiquitin-conjugating enzymes (UBCs), and another family member—Apollon—contains a UBC domain. These IAP family members could be involved in the ubiquitylation of themselves and associated proteins.

Originally characterized as a potent inhibitor of proteolytic caspase activity, XIAP can directly bind to and inhibit caspases 3, 7, and 9 [9,10]. This IAP functions as an E3 ubiquitin ligase for pro-apoptotic proteins such as caspases, second mitochondrial-derived activator of caspase (SMAC) and ARTS

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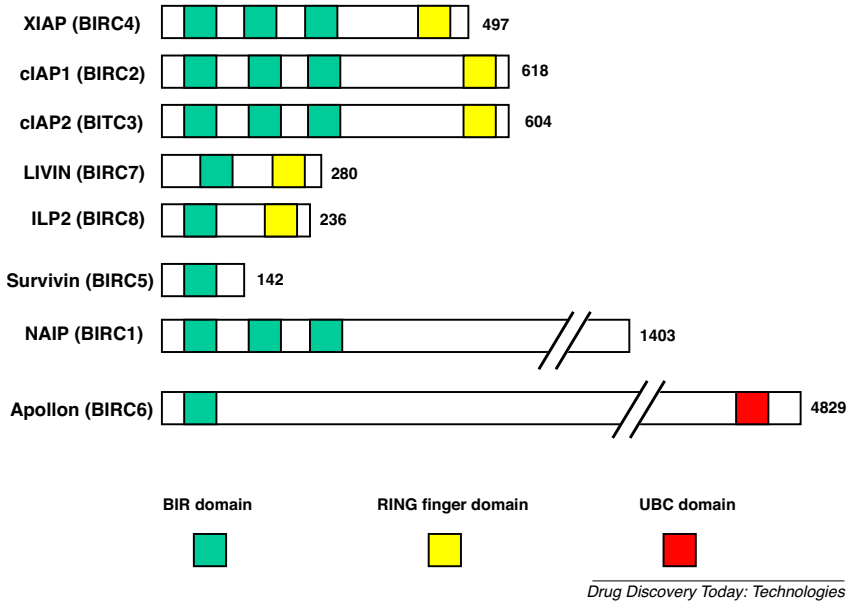

Fig. 1. Human inhibitor of apoptosis protein (IAP) family proteins. The number on the right represents the number of amino acids in the full-length proteins.

[11–13], and also plays a role in nucleotide-binding oligomerization domain-containing (NOD) signaling and immune responses through the ubiquitylation of receptor-interacting serine/threonine-protein kinase 2 (RIPK2) [14,15]. Gene targeting studies have demonstrated that while XIAP-deficient mice do not show obvious abnormalities during development, they show subtle phenotypes in their sensitivity to cell death [16–18]. This suggests that XIAP inhibition is unlikely to cause serious adverse effects in normal tissues. However, overexpression of XIAP is characteristic of many cancers. This overexpression is involved in resistance to cancer therapy, implying that XIAP could be a promising target for cancer therapy [7,19–21].

Although cIAP1 and cIAP2 are structurally similar to XIAP and can bind to caspases, they are poor caspase inhibitors [6]. Instead, cIAP1 and 2 regulate various signaling pathways including nuclear factor kappa-light-chain-enhancer of activated B cell (NFκB) pathways [22–26]. Following stimulation of tumor necrosis factor-α (TNFα), multiple signaling proteins are recruited to the TNF receptor (TNFR), including cIAP1 and 2. Subsequently, the E3 ligase activity of cIAP1/2 is stimulated, where the K63-linked polyubiquitylation of receptor-interacting serine/threonine-protein kinase 1 (RIP1) and cIAP1/2 themselves is formed, resulting in the recruitment of various signaling mediators including transforming growth factor beta-activated kinase 1 (TAK1)/TAK1-binding protein 1 (TAB1 TAB1)/TAB2 and the linear ubiquitin chain assembly complex (LUBAC). The LUBAC-mediated ubiquitylation of RIP1 and TNFR1 leads to efficient recruitment and activation of IκB kinase (IKK) (composed of NFκB essential modulator [NEMO]/IKKα/IKKβ), which in turn activates NFκB [24,27]. Inhibition or degradation of cIAP1/2 leads to the formation of different TNFR signaling complexes which activate caspase 8-mediated apoptosis or RIP3-mediated necroptosis [28,29]. In B cell lineage, B-cell receptor signaling stimulates the caspase recruitment domain-containing protein 11 (CARD11)-mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1)-B-cell CLL/lymphoma 10 protein (BCL10) adaptor complex to activate NFκB, with the concomitant cIAP1/2-mediated K63-linked ubiquitylation of BCL10 and cIAP1/2 themselves. Recruitment of IKK and LUBAC follows [30]. Deletion of both cIAP1 and cIAP2 in B cells indicates an essential role of cIAP1/2 in regulating B cell survival and responsiveness [31]. cIAP1/2 is also involved in the alternative NFκB pathway by mediating K48 polyubiquitylation and degradation of NIK [32–35]. Thus, cIAP1/2 play a pivotal role in mediating NFκB signaling pathways, the deregulation of which can result in various diseases including cancer and inflammation. Overexpression of cIAP1 has been observed in various cancers including cervical, esophageal, hepatic and lung cancers, and is involved in the resistance to therapy [36–39].

Apollon—which contains a UBC domain and inhibits apoptosis by regulating caspases—is reported to be an independent poor prognostic factor in childhood leukemia [40–42]. Another IAP family member, Livin, is not expressed in most normal differentiated tissues, but is present in several cancers including breast, gastric, colon and pancreatic cancers as well as melanomas [43,44]. Thus, IAPs are expressed in a variety of cancers, which makes them intractable. In this review article, we focus on the development of SNIPERs that exploit the ubiquitin ligase activities of IAPs to induce degradation of target proteins.

Induction of cIAP1 degradation by MeBS

Bestatin is an aminopeptidase inhibitor isolated from actinomycetes [45,46]. We previously reported that bestatin methyl-ester (MeBS) increases the sensitivity of cancer cells to apoptosis induced by various stimuli, such as anti-cancer drugs and death receptor ligation, as well as causing a reduction in cellular cIAP1 levels [47]. Mechanistic analysis has demonstrated that MeBS interacts with the third BIR domain of cIAP1, and induces RING-mediated auto-ubiquitylation and proteasomal degradation of cIAP1 (Fig. 2a).

Studies of the structure-activity relationship of MeBS showed that the reduction of cIAP1 expression was maintained when the esterified methyl residue of the carboxylic acid was substituted for more bulky residues. However, modification of the bestatin backbone seriously affected the activity in many cases [47]. These observations indicate that the bestatin backbone of MeBS interacts with cIAP1, while the esterified methyl residue is not involved in the interaction. Based on these observations, we planned to develop a series of chimeric compounds by substituting the methyl residue with the ligand for a particular target protein. The resulting molecule may be able to effectively cross-link the target protein and cIAP1, enabling cIAP1-mediated ubiquitylation and proteasomal degradation of the target protein (Fig. 2b).

Development of 1st generation SNIPERs

Through collaboration with medicinal chemistry groups, we developed a number of chimeric molecules composed of a target ligand and bestatin. These molecules were termed Specific and Nongenetic IAP-dependent Protein Erasers (SNIPERs). By incorporating all-trans retinoic acid (ATRA), 4-hydroxy tamoxifen (4-OHT), androgen antagonists, Abelson murine leukemia (ABL) kinase inhibitors, KHS compounds and alkyl chloride into SNIPER molecules as ligands, successful targeting and degradation of cellular retinoic acid binding protein (CRABP-II) [48–52], estrogen receptor-α (ERα) [53,54], androgen receptor (AR) [55,56], oncogenic fusion BCR-ABL protein [57,58], transforming acidic coiled-coil-3 (TACC3) [59] and Halo-tag proteins [60], respectively, by SNIPERs was achieved within cells (Table 1 and Fig. 3a).

The degradation of target proteins by these SNIPERs is highly specific, and a SNIPER against ERα (SNIPER[ER]) selectively induces necrotic cell death in ERα-expressing breast cancer cells [54]. A SNIPER against TACC3 induces apoptotic cell death in cancer cells that express large amounts of TACC3 compared with normal fibroblasts [59]. These results highlight the potential of SNIPERs in cancer therapy. However, bestatin-based 1st generation SNIPERs are only able to induce degradation when administered at concentrations of 10 μM or higher, therefore the devel-

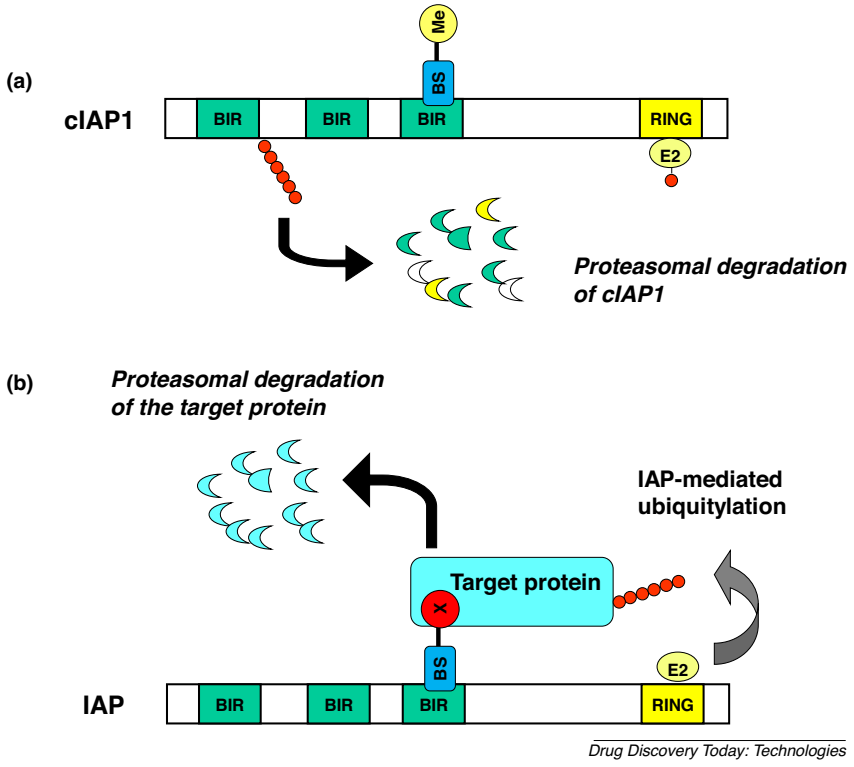

Fig. 2. (a) Mechanism of cIAP1 degradation induced by MeBS. (b) Mechanism of IAP-mediated ubiquitylation and degradation of target proteins induced by SNIPERs.

opment of novel SNIPERs that can induce degradation at lower concentrations was required.

Development of 2nd generation SNIPERs

The incorporation of a ligand with higher binding affinity was considered in order to improve the protein-knockdown activity of SNIPER molecules. Because the affinities of ligands to their respective target proteins are typically much higher than that of MeBS to cIAP1, substitution of the bestatin module with such ligands was trialed to improve the degradation activity of SNIPERs. Indeed, substituting an IAP antagonist MV1 for bestatin improved the degradation of target proteins such as CRABP-II [48,52], BCR-ABL [58], ERα [61] and His-tag proteins [62,63] (Table 1 and Fig. 3b).

To further improve the activity of the SNIPER(ER), we tested various combinations of ERα ligands and IAP ligands, and optimized the linker. The molecule SNIPER(ER)-87 was developed, composed of 4-OHT (ERα ligand) and an LCL-161 derivative (IAP ligand) with a polyethylene glycol (PEG) linker (Fig. 3c) [61]. The SNIPER(ER)-87 induced IAP-mediated ubiquitylation and proteasomal degradation of ERα at nano-molar concentrations, which is 1000-times lower than the effective concentrations of the bestatin-based 1st generation SNIPER(ER)s. Mechanistically, SNIPER(ER)-87 preferentially recruited XIAP rather than cIAP1 to ERα, and XIAP was the major ubiquitin ligase responsible for the degradation of ERα. The ERα-degradation activity of SNIPER(ER)-87 correlates well with its anti-tumor activity against breast cancer cells that show estrogen-dependent growth. This SNIPER has also been shown to have protein knockdown and anti-tumor activities in a tumor xenograft model where MCF-7 human breast cancer cells were transplanted to nude mice [61].

Table 1. Target proteins successfully degraded by SNIPERs.

Target protein	Target ligand	IAP ligand	Degradation	Ref
Estrogen receptor	4-OHT	Bestatin	+	[53,54]
		MV1	++	[61]
		LCL	+++	[61]
		Other IAP antagonists	+++	[64]
	Estron	Bestatin	+	[55]
	Peptide ligand	MV1	+	[75]
Androgen receptor	AR antagonists	Bestatin, LCL	+	[56]
	DHT	Bestatin	+	[55]
BCR-ABL	imatinib	Bestatin	+	[57,58]
	HG-7-85-01	MV1	+	[58]
	HG-7-85-01	LCL	++	[58]
	Dasatinib	MV1	++	[58]
	Dasatinib	LCL	+++	[58,65]
	ABL001	LCL	+++	[66]
CRABP-II	ATRA	Bestatin	+	[48–52]
		MV1	++	[48,52]
RARα	Ch55	Bestatin	+	[55]
TACC3	KHS-108	Bestatin	+	[59]
BRD4	JQ1	LCL	+++	[61]
PDE4	PDE inhibitor	LCL	+++	[61]
Notch1	Peptide ligand	LCL	+	[76]
huntingtin	BTA, PDB	Bestatin	+	[77]
His-tag	Ni-NTA	MV1	+	[62,63]
Halo-tag	Alkyl-Cl	Bestatin	+	[60]

LCL in the IAP ligand column represents the LCL-161 derivative. +, ++ and +++ in the Degradation column represent the DC_{50} values ≥ 1 μM, ≥ 100 nM and < 100 nM, respectively.

Potent SNIPERs against BCR-ABL, bromodomain-containing protein 4 (BRD4) and phosphodiesterase type 4 (PDE4) have been developed using the LCL-161 derivative as an IAP ligand, which induce the degradation of respective target proteins at nano-molar concentrations [58,61,64–66] (Table 1). This highlights the potential of including high

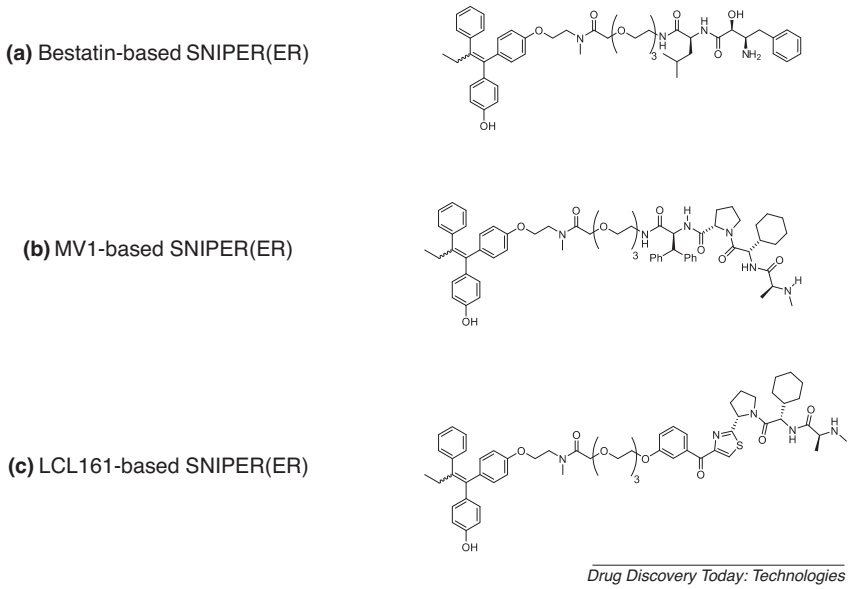

Fig. 3. Chemical structure of the (a) bestatin-based, (b) MV1-based, and (c) LCL161-based SNIPER(ER)s.

affinity IAP antagonists into SNIPERs to develop potent SNIPERs against various target proteins.

Significance of IAP degradation in anticancer activity

Unlike chimeric molecules that recruit Cullin-based ubiquitin ligase complexes including VHL and CRBN, SNIPERs induce degradation of the ubiquitin ligases cIAP1 and XIAP [61,64,67]. Degradation of cIAP1 is robustly triggered by the binding of SNIPERs to cIAP1, which is reminiscent of the IAP antagonist-induced cIAP1 degradation [68,69]. However, degradation of XIAP requires the formation of a ternary complex (XIAP-SNIPER-target protein), as does the degradation of the target protein [67]. It is probable that XIAP and the target protein are simultaneously ubiquitylated only when specific targets interact with XIAP and induce conformational changes that expose the lysine residue of XIAP to E2-UBC. Alternatively, multiple XIAP proteins are recruited to oligomeric targets, which may induce trans-ubiquitylation of XIAP, though we do not have any evidences. Interestingly, the pharmacological hook effect is observed for target proteins but not for XIAP. This could be explained by the multiple target proteins existing with a wide range of binding affinities to the target ligand. At lower concentrations, SNIPERs will preferentially bind to high-affinity target proteins, forming a ternary complex with XIAP, which results in degradation of the high affinity target and XIAP. At higher concentrations, however, the SNIPER molecules begin to interact with low affinity targets, reducing the amount of XIAP in the ternary complexes with high affinity targets. Accordingly, the degradation of high affinity targets is suppressed while XIAP degradation remains unchanged as illustrated in Figure 7 of Reference [67].

Thus, SNIPERs induce simultaneous degradation of the target proteins with cIAP1 or XIAP, although the latter is degraded less efficiently than cIAP1. The degradation of the ubiquitin ligases themselves could be expected to result in inefficient degradation of the target proteins. However, significant amounts of the IAPs are constantly synthesized in the cells, which will contribute to the degradation of target proteins. In addition, other IAP family members may play a role. Therefore, we hypothesize the target degradation could be maintained in cells treated with SNIPERs, despite the SNIPER-induced degradation of some IAP family proteins.

The degradation of IAPs can also have positive effects, as cancer cells often overexpress IAP family proteins with a concomitant increase in resistance to cancer therapy reported [7,19–21,36–39]. Downregulation of IAPs sensitizes cancer cells to apoptosis induced by various stimuli, and IAPs have therefore been highlighted as promising therapeutic targets [7,19,70,71]. Currently, several antagonists that may inhibit IAP function are under clinical development. Because SNIPERs contain an IAP antagonist module, they could be exploited for the efficient killing of cancer cells. This has been tested in MCF7 breast cancer cells that require IAP activity to survive, where SNIPER(ER)-105, -110 and -126 have been reported to cause increased degradation of ERα, cIAP1 and XIAP compared with SNIPER(ER)-87. Their higher affinity for the target proteins results in increased induction of apoptotic cell death [64].

Future prospect of the chemical protein knockdown

The majority of drugs that have been developed recently to target specific molecules are antibodies or small molecule enzyme inhibitors such as kinase inhibitors. Although they show excellent therapeutic activities in clinic, it is speculated that only 25%–30% of known cellular proteins are able to be targeted by these modalities. The remaining 70%–75% of proteins are regarded as ''undruggable'', and include intracellular proteins without enzymatic activity such as scaffold proteins and transcription factors. These proteins could be targeted for degradation by the activities of SNIPERs and PROTACs. The only essential feature required for a ligand being integrated into a SNIPER is the ability to bind to the target, meaning that compounds with no or insufficient inhibitory activity can be used. In the case of proteins with multiple domains, a ligand against any domain can be used to develop a SNIPER, which would increase the options for the development of novel drugs targeting a single protein. Resistance to kinase inhibitors is often attributed to mutations in the kinase domain, therefore this approach could be used as an alternative strategy to overcome drug resistance, by capturing different domains of oncogenic kinases to induce their degradation.

Pharmacologically, degradation provides additional beneficial effects compared with inhibition of the target protein [72,73]. Both the degradation and inhibition of the oncogenic kinase BCR-ABL result in an anti-tumor effect, but the effects of degradation are sustained when the drugs are removed following short-term treatment [65]. Treatment with kinase inhibitors would allow immediate restoration of the kinase signaling initiated by BCR-ABL when the drug was removed, while the signaling remains suppressed in cells treated with SNIPER(ABL) until a significant amount of BCR-ABL protein is synthesized and protein function is recovered. Consistently, chronic myelogenous leukemia (CML) cells treated with kinase inhibitors resume proliferation immediately after drug removal, whereas cells treated with SNIPER (ABL) could not proliferate and eventually underwent apoptotic cell death under the same conditions (Fig. 4). Thus, degradation may induce longer lasting effects compared with target protein inhibition.

Currently, the ubiquitin ligases that can be recruited to the target proteins by chimeric small molecules include IAPs, VHL, CRBN and MDM2. However, recruitment of ubiquitin ligases does not always result in efficient degradation of the target proteins. The efficiency of the target degradation

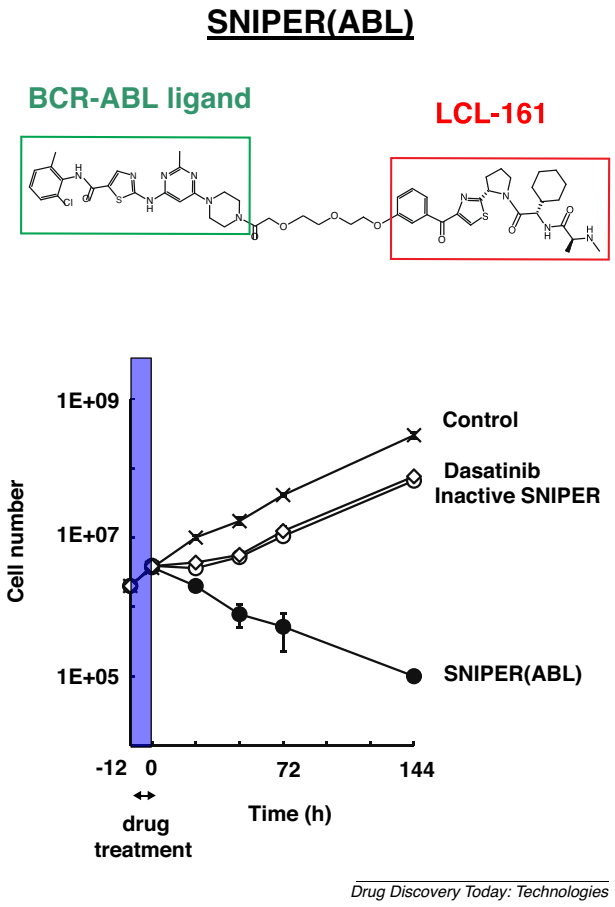

Fig. 4. Proliferation of chronic myelogenous leukemia K562 cells after treatment with SNIPER(ABL) and dasatinib for 12 h. SNIPER(ABL) shows a long-lasting growth inhibition compared with kinase inhibitor. Modified from ref under the CC BY license (Creative Commons Attribution v4.0 International License).

depends on the combination of the target and E3 ligases. In our experiments, when dasatinib was used as a ligand to target the degradation of BCR-ABL, the recruitment of IAPs resulted in improved degradation compared with the recruitment of VHL or CRBN. However, when HG-7-85-01 was used as a BCR-ABL ligand, recruitment of VHL showed better activity than that of IAP and CRBN [65]. Thus, optimization of the ligand and E3 ligase combination is important for the development of a chimeric molecule with potent degradation activity. It is also important to develop novel ligands that can recruit different E3 ubiquitin ligases, since there are more than 600 ubiquitin ligases in the cells that could be recruited to induce degradation.

Conclusion

It has been almost two decades since the concept of targeted protein degradation using a bifunctional molecule was proposed [74]. Recent progress on the identification of ligands that recruit ubiquitin ligases to target proteins makes this technology a feasible and very attractive approach for the development of novel drugs against currently undruggable target proteins. The SNIPERs recruit IAP ubiquitin ligases, which are a family of anti-apoptotic proteins that are frequently overexpressed in cancers. Because the level of IAP expression correlates with the degree of resistance to cancer therapy, IAPs are regarded as promising therapeutic targets against cancers, and several IAP antagonists are currently under clinical evaluation. The structures of SNIPERs contain IAP antagonist modules that recruit IAPs, and they potently induce the degradation of target proteins as well as the IAPs themselves. This is an efficient method of killing cancer cells that require IAPs for survival. Therefore, protein degradation by SNIPERs is a promising strategy for the development of anti-cancer drugs.

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