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# Staurosporine and venetoclax induce the caspase-dependent proteolysis of MEF2D-fusion proteins and apoptosis in *MEF2D*-fusion (+) ALL cells

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K252a (PubChem CID: 3035817)
MG-132 (PubChem CID: 462382)
MG-262 (PubChem CID: 490002)
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ABSTRACT

*MEF2D*-fusion (*M-fusion*) genes are newly discovered recurrent gene abnormalities that are detected in approximately 5 % of acute lymphoblastic leukemia (ALL) cases. Their introduction to cells has been reported to transform cell lines or increase the colony formation of bone marrow cells, suggesting their survival-supporting ability, which prompted us to examine M-fusion-targeting drugs. To identify compounds that reduce the protein expression level of MEF2D, we developed a high-throughput screening system using 293T cells stably expressing a fusion protein of MEF2D and luciferase, in which the protein expression level of MEF2D was easily measured by a luciferase assay. We screened 3766 compounds with known pharmaceutical activities using this system and selected staurosporine as a potential inducer of the proteolysis of MEF2D. Staurosporine induced the proteolysis of M-fusion proteins in *M-fusion* (+) ALL cell lines. Proteolysis was inhibited by caspase inhibitors, not proteasome inhibitors, suggesting caspase dependency. Consistent with this result, the growth inhibitory effects of staurosporine were stronger in *M-fusion* (+) ALL cell lines than in negative cell lines, and caspase inhibitors blocked apoptosis induced by staurosporine. We identified the cleavage site of MEF2D-HNRNPUL1 by caspases and confirmed that its caspase cleavage-resistant mutant was resistant to staurosporine-induced proteolysis. Based on these results, we investigated another Food and Drug Administration-approved caspase activator, venetoclax, and found that it exerted similar effects to staurosporine, namely, the proteolysis of M-fusion proteins and strong growth inhibitory effects in *M-fusion* (+) ALL cell lines. The present study provides novel insights into drug screening strategies and the clinical indications of venetoclax.

## 1. Introduction

Target-based screening is a major approach in recent anti-cancer drug development. The target molecule of a certain cancer is initially selected based on our knowledge of molecular pathology. Compounds are screened by evaluating their inhibitory effects on the activity of a target molecule or affinity to the target molecule using an *in vitro* assay system as well as their cytotoxic effects using cell lines. Although this is a powerful method, target molecules are restricted to enzymes, mainly kinases, and cell surface proteins in most cases because technical know-how for drug development is unevenly distributed to the synthesis of kinase inhibitors and production of antibodies. Intracellular non-enzymatic proteins, such as transcription factors and scaffold proteins, are recognized as undruggable targets; however, aberrant transcription factors, particularly the fusion proteins of transcription factors, are major causes of cancers and promising molecular targets of anti-cancer drugs. Therefore, methods to develop drugs that target undruggable targets have long been awaited.

The myocyte enhancer factor 2 (MEF2) family of transcription factors consists of 4 members in mammalian cells (MEF2A, 2B, 2C, and

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2D). These members were originally identified as major transcriptional activators of muscle differentiation [1,2], but were subsequently shown to be involved in the differentiation of neuronal cells and lymphoid cells [3,4]. MEF2 family members, particularly MEF2D, have also been implicated in cancer biology. The overexpression of MEF2D was previously reported in hepatocellular carcinoma and gastric cancer, correlated with the prognosis of these cancers, and promoted the proliferation of cancer cells [5,6]. Moreover, we and others recently identified *MEF2D*-fusion (*M-fusion*) genes in approximately 5% of B-cell acute lymphoblastic leukemia (ALL) cases [7–9]. MEF2D is the 5′ partner in all described fusions, whereas B-cell CLL/lymphoma (BCL) 9 and heterogeneous nuclear ribonucleoprotein U-like 1 (HNRNPUL1) are the two major 3′ partners in approximately 90 % of fusions. We also previously found that the loss of the micro RNA target site in the wild-type *MEF2D* gene by translocation led to the strong expression of M-fusion proteins in ALL cells by evading micro RNA [10]. These findings prompted us to examine drugs that target M-fusion proteins as possible therapeutic agents for *M-fusion* (+) ALL.

The primary purpose of the present study is the establishment of a new high-throughput screening system that detects reductions in target proteins to make undruggable targets druggable. Accordingly, we developed a high-throughput screening system to identify drugs that induce reductions in M-fusion proteins and discovered staurosporine as an inducer of the proteolysis of M-fusion proteins. Secondary, we attempted to identify Food and Drug Administration (FDA)-approved drugs that induce reductions in M-fusion proteins. Since caspases cleaved M-fusion proteins under the staurosporine treatment, we selected another caspase activator, venetoclax, as a potential therapeutic agent for *M-fusion* (+) ALL. Venetoclax induced the proteolysis of M-fusion proteins and exerted strong growth inhibitory effects in *M-fusion* (+) ALL cell lines. The present results provide novel insights into drug screening strategies and the clinical indications of venetoclax, which is currently approved for the treatment of chronic lymphoid leukemia (CLL) and acute myeloid leukemia (AML).

## 2. Materials and methods

### 2.1. Cells and cell culture

293T, a renal cell cancer cell line, was maintained in DMEM supplemented with 10 % fetal bovine serum (FBS). The *BCR-ABL* (+) ALL cell lines, ALL-1 and NPhA1, and Daudi, a Burkitt lymphoma cell line, were maintained in 10 % FBS-containing RPMI1640 and Iscove, respectively [11,12]. Kasumi-7 and Kasumi-9 were obtained from the Japanese Collection of Research Bioresources Cell Bank, and maintained in RPMI1640 supplemented with 10 % FBS. TS-2 was established by Dr. M Imaizumi [13], kindly provided through the Tokyo Medical and Dental University Bioresource Research Center (TMDUBR), and maintained in RPMI supplemented with 10 % FBS. None of the cell lines were authenticated in our laboratory; however, the existence of M-fusion genes in Kasumi-7, Kasumi-9, and TS-2 was confirmed by Sanger sequencing.

### 2.2. Reagents and antibodies

Staurosporine, a Bax channel blocker (inhibitor of mitochondrial apoptosis-induced channel 1, iMAC1), epoxomicin, bortezomib, bafilomycin A1 (BafA1), 3-methyladenine (3-MA), and cytarabine were purchased from Cayman Chemical (Ann Arbor, MN). K252a was from BioVision, Inc. (Milpitas, CA). Venetoclax was from LC Laboratories (Woburn, MA). MG-262 was from Adipogen (San Diego, CA). Vincristine was from Eli Lilly Japan (Kobe, Japan). Cycloheximide and MG-132 were from Sigma-Aldrich Japan (Tokyo, Japan). Z-VAD-FMK was from ENZO Life Sciences (Farmingdale, NY).

An anti-MEF2D-N antibody, the antigen of which was the N terminus of MEF2D, and an anti-firefly luciferase antibody (EPR17790) were from Abcam (Cambridge, MA). Anti-cleaved caspase-3, anti-cleaved caspase-7, and anti-cleaved PARP were from Cell Signaling Technology (Beverly, MA). An anti-human CD19 antibody was purchased from BD Biosciences (San Jose, CA, USA). Recombinant caspase-3 and recombinant caspase-7 were purchased from R&D Systems (Minneapolis, MN).

### 2.3. Plasmids

Luc/pIRES-EGFP was made by inserting the cDNA of firefly luciferase cut from pGL4 20.1 (Promega) into the pIRES-EGFP vector (Promega) at the Sal I and BamH I sites. Mutations to make Sal I and BamH I sites just before and after the coding region of luciferase, respectively, were introduced by PCR mutagenesis into pGL4 20.1 in advance. MEF2D N-Luc/pIRES-EGFP was made by inserting the cDNA of MEF2D N (see the Results section) cut from MEF2D/pcDNA into Luc/pIRES-EGFP at the EcoR I and Sal I sites. The mutation to make a Sal I site just after the coding region of MEF2D N was introduced by PCR mutagenesis into MEF2D/pcDNA in advance. Flag-MEF2D-HNRNPUL1/pcDNA was described previously [10]. MEF2D-HNRNPUL1-HiBiT/pCDNA was made by inserting the HiBiT-tag sequence (33 bp) just before the stop codon by PCR mutagenesis.

### 2.4. Establishment of stable transfectants

MEF2D N-Luc/pIRES-EGFP and Luc/pIRES-EGFP were introduced into 293T using nucleofector (Lonza, Wuppertal, Germany) according to the manufacturer's instructions. Stable transfectants were established *via* a selection culture with 1200 μg/mL of geneticin (Thermo Fisher Scientific, Waltham, MA) and the sorting of GFP-positive cells, and were designated as MEF2D N-Luc/293T and Luc/293T, respectively.

### 2.5. Compound library

A validated library consisting of 3398 off-patent drugs and pharmacologically active reagents was kindly provided by the Drug Discovery Initiative (the University of Tokyo, Tokyo, Japan). This library is also commercially available as Prestwick and Lopack chemical libraries by Prestwick Chemical (Strasbourg, France) and Sigma-Aldrich (St. Louis, MO), respectively. Detailed information is available on their web sites: http://www.prestwickchemical.com/index.php?pa=26 and http://www.sigmaaldrich.com/catalog/product/sigma/lo1280?lang=en&region=US, respectively. The SCADS inhibitor kit consisting of 398 molecular targeting inhibitors was generously provided by the Screening Committee of Anticancer Drugs supported by Scientific Support Programs for Cancer Research from MEXT (Tokyo, Japan).

### 2.6. Screening method

MEF2D N-Luc/293T and Luc/293T ($1 \times 10^4$/well) were seeded on 96-well plates. The next day, the library compounds (2 μM each) were added to each well. After a 24 h exposure to these compounds, luciferase activity in each well was measured using the ONE-Glo™ luciferase assay system (Promega) and the Glo Max Navigator® microplate luminometer (Promega).

### 2.7. Immunoblotting and HiBiT blotting

Immunoblotting was performed as described previously [14]. All immunoblots were performed at least two times. The intensity of the observed bands was quantified in some experiments. The Nano Glo HiBiT blotting system (Promega) was used to visualize HiBiT-tagged proteins after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotting on PVDF membranes.

### 2.8. Viable cell numbers and apoptosis assays

Viable cell numbers were measured by the MTT assay using the Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan) according to the manufacturer's instructions. Apoptosis was evaluated by detecting the Annexin V-positive and DAPI-negative fractions using flow cytometry. DAPI and Annexin-V-FLUOS Staining Kits were obtained from Life Technologies (Carlsbad, CA) and Roche Applied Science (Penzberg, Germany), respectively.

### 2.9. In vitro caspase cleavage assay

Unmutated and mutated M-H were synthesized *in vitro* using the TNT transcription/translation system (Promega) with [$^{35}$S]-Met labeling according to the manufacturer's instructions. One microliter of synthesized M-H was incubated at 37 ˚C for 4 h with 20 ng of recombinant caspase-3 or caspase-7 in 10 μl of reaction buffer [25 mM HEPES (pH 7.5), 0.1 %(w/v) CHAPS, and 1 mM dithiothreitol]. The reaction mixture was subjected to 15 % SDS-PAGE followed by an image analysis using a BAS 2000 image analyzer (Fuji Film, Tokyo, Japan).

### 2.10. Statistical analysis

Differences between two groups were analyzed with the Student's *t*-test (two-tailed). Statistical analyses were performed using statflex ver.6 (https://www.statflex.net/) and differences with p values less than 0.05 were considered to be significant. Values are presented as the mean ± standard error of the mean (SEM).

## 3. Results

### 3.1. Staurosporine and its analog were selected as compounds that reduced MEF2D protein levels

To develop a high-throughput screening system for compounds that reduce the protein expression levels of MEF2D, we established a cell line for screening in which the protein expression level of MEF2D was measurable using the luciferase assay. The expression vector for the fusion protein of the N-terminal portion of MEF2D comprising amino acids (aa) 1–203 (MEF2D N) and luciferase (MEF2D N-Luc) was transfected into 293T cells, and the stable transfectant was established (MEF2D N-Luc/293T). Although several break points in *MEF2D* have been reported for *M-fusion* genes, MEF2D N is commonly included in all M-fusion proteins. Stable transfectants of the expression vector for luciferase were also established (Luc/293T). The expression of MEF2D N-Luc and luciferase in stable transfectants was confirmed by immunoblotting with an anti-luciferase antibody (Supplemental Fig. S1A) and luciferase assay (Supplemental Fig. S1B). We seeded MEF2D N-Luc/293T on 96-well plates, added compounds, and performed the luciferase assay to measure the protein expression levels of MEF2D N-Luc (Fig. 1A). The coefficient variation value of this system was 4.7 %, demonstrating the low measurement error of this system. We were unable to estimate the Z'-factor at the time of the development of the system because we did not have a positive control for the proteolysis inducer of MEF2D. We screened 3766 compounds, including FDA-approved drugs and compounds with known pharmaceutical activities, using this system. Since cycloheximide, a known inhibitor of protein synthesis, produced a relative luciferase value of 0.5, we set it as the threshold for hit compounds. Seventeen compounds produced relative luciferase values of less than 0.5 (Fig. 1B), and were used in the second screening, in which they were added to Luc/293T and their ability to induce luciferase reductions was examined in order to exclude compounds whose proteolytic targets were luciferase. Staurosporine, a multi-kinase inhibitor, and K252a, an analog of staurosporine, strongly reduced relative luciferase values in MEF2D N-Luc/293T, but not in Luc/293T (Fig. 1C). Strong dose-dependent reductions in MEF2D N-Luc by these compounds were observed by immunoblotting (Fig. 1D and E). Although relatively weak reductions in luciferase were also observed, they were consistent with the reductions induced in the viable cell numbers of the screening cell lines by the treatment. These compounds were selected as hit compounds. The structures of staurosporine and K252a are shown in Supplemental Fig. S1C. A strong discrepancy was observed between the expression of α-tubulin, which was used as the loading control of immunoblots, and the MTT value reflecting viable cell numbers (Fig. 1D and E), suggesting that these compounds cause the proteolysis of α-tubulin in addition to MEF2D. We attempted to use β-actin as an alternative loading control, but obtained similar results (data not shown).

### 3.2. Staurosporine reduced M-fusion protein levels in ALL cells in dose-dependent manners

We examined whether staurosporine and K252a reduced M-fusion protein levels in *M-fusion* (+) ALL cell lines. We previously found that Kasumi-7 and Kasumi-9 had *MEF2D-HNRNPUL1 (M-H)* [7], while others reported that TS-2 had *MEF2D-DAZAP1 (M-D)* [15]. Staurosporine depleted M-H in Kasumi-7 and Kasumi-9, and M-D in TS-2 in a dose-dependent manner. A concentration of 1 μM was sufficient to deplete M-fusion proteins after a 6-h treatment with staurosporine (Fig. 2A). K252a also induced dose-dependent reductions in M-fusion protein levels; however, these effects were weaker than those of staurosporine (Fig. 2B). A discrepancy was also observed between α-tubulin expression and live cell numbers in these experiments. Although relative live cell numbers were more than 80 % in all three cell lines after the 6-h treatment with 1 μM staurosporine, α-tubulin reduced them to 54 %, 37 %, and 28 % in Kasumi-7, Kasumi-9, and TS-2, respectively (Supplemental Fig. S2A). Similar results were obtained with the K252a treatment (Supplemental Fig. S2B). These results also indicated that these compounds induced the proteolysis of α-tubulin. We selected staurosporine for further analyses and disregarded the results obtained from immunoblots with the anti-α-tubulin antibody when staurosporine was used. We used staurosporine in an immunoblot analysis at a concentration less than or equal to 1 μM within the 6-h treatment. In this condition, the viable cell number of the treated cells were expected to be more than 80 % of the control cells.

### 3.3. Growth inhibitory effects of staurosporine and its analog were stronger in M-fusion (+) cell lines than in M-fusion (−) cell lines

Staurosporine is a multi-kinase inhibitor that inhibits growth and induces apoptosis in many tumor cells, including M-fusion (-−) tumor cells. We hypothesized that M-fusion proteins support the survival of *M-fusion* (+) ALL cells, and M-fusion protein reductions by staurosporine increased its cytotoxic effects. We investigated whether *M-fusion* (+) ALL cell lines were more sensitive to staurosporine than *M-fusion* (−) cell lines. We examined the growth-inhibitory effects of staurosporine in 3 *M-fusion* (+) ALL cell lines (Kasumi-7, Kasumi-9, and TS-2), 2 *M-fusion* (−) ALL cell lines (ALL1 and NPhA1), and a Burkitt lymphoma cell line (Daudi). As expected, staurosporine exerted stronger growth inhibitory effects in *M-fusion* (+) ALL cell lines than in *M-fusion* (−) cell lines. The mean $GI_{50}$ observed in *M-fusion* (+) ALL cell lines and *M-fusion* (−) cell lines were 55 and 574 nM, respectively (Fig. 3A). We also investigated the growth inhibitory effects of K252a in the same cell lines. Although they were generally weaker than those of staurosporine, growth inhibitory effects were stronger in *M-fusion* (+) ALL cell lines than in *M-fusion* (−) cell lines. The mean $GI_{50}$ observed in *M-fusion* (+) ALL cell lines and *M-fusion* (−) cell lines were 306 nM and 6.1 μM, respectively (Fig. 3B). On the other hand, the growth inhibitory effects of other conventional anti-tumor drugs used to treat ALL, such as cytarabine and vincristine, were not markedly different between *M-fusion* (+) ALL cell lines and *M-fusion* (−) cell lines. The mean $GI_{50}$ in *M-*

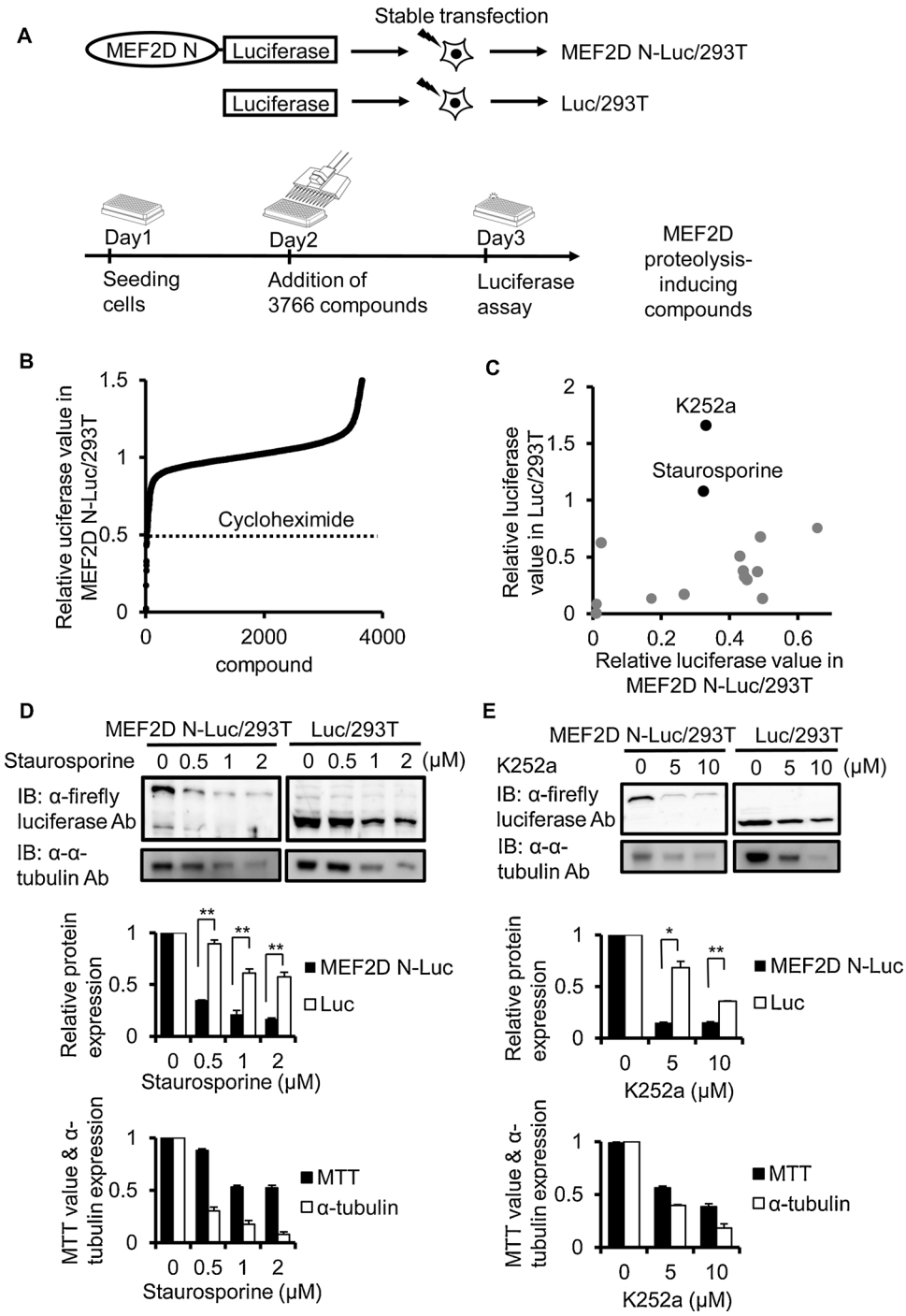


**Fig. 1.** Staurosporine and its analog were selected as compounds that induced reductions in the MEF2D protein. (A) Schema of the screening system. Compounds were screened using the system in which the expression level of the MEF2D protein was measured by the luciferase assay. (B) Results of the 1st screening. The relative luciferase values of 3766 compounds in the 1st screening using MEF2D N-Luc/293T were plotted on a scattergram. Relative luciferase values are relative to control values in DMSO-treated cells. (C) Results of the 2nd screening. MEF2D N-Luc/293T and Luc/293T were treated with the top 17 compounds from the 1st screening. The luciferase values of the compounds were plotted on a scattergram, on which the relative luciferase values in MEF2D N-Luc/293T and Luc/293T were set on the X-axis and Y-axis, respectively. (D)-(E) Staurosporine and K252a reduced MEF2D N-Luc more than luciferase in 293T. MEF2D N-Luc/293T and Luc/293T were treated with staurosporine (D) or K252a (E) at the indicated concentration for 24 h. Two sets of cells were prepared. One set of cells was lysed and subjected to immunoblotting with the indicated antibodies. Another set was subjected to the MTT assay. The intensity of the observed bands in immunoblots was quantified and plotted on the bar charts as the mean value of three independent experiments (middle panel). Error bars indicate the SEM. Differences in protein expression levels between MEF2D N-Luc and Luc at each concentration were statistically tested. **: $p < 0.01$, *: $p < 0.05$. The relative MTT value and relative expression of α-tubulin from immunoblots were plotted on the bar charts as the mean value of three independent experiments from MEF2D N-Luc/293T (bottom panel).

*fusion* (+) ALL cell lines *vs*. *M-fusion* (−) cell lines were 2.05 μM *vs*. 4.04 μM for cytarabine and 2.62 μM *vs*. 3.47 μM for vincristine (Supplemental Fig. 3A and B). These results suggested that staurosporine-induced M-fusion protein reductions enhanced its growth inhibitory effects in *M-fusion* (+) ALL cells.

### 3.4. Caspase inhibitors inhibited staurosporine-induced M-fusion protein reductions and apoptosis

Since staurosporine is a multi-kinase inhibitor and MEF2D is phosphorylated by several kinases, such as cyclin-dependent kinase 5 (Cdk5) [16] and extracellular signal-regulated kinase 5 (ERK5) [17], we hypothesized that staurosporine alters the phosphorylation status of MEF2D and, thus, induces the proteasome-dependent degradation of MEF2D; therefore, we investigated whether various proteasome inhibitors inhibited staurosporine-induced M-fusion protein reductions. We tested MG-132, epoxomicin, MG-262, and bortezomib; however, inhibition was not observed (Supplemental Fig. 4A–D). Since MEF2D expression levels were previously reported to be regulated by chaperon-mediated autophagy [18], we examined the involvement of autophagy in staurosporine-induced M-fusion protein reductions using BafA1 and 3-MA, which are autophagy inhibitors, but did not observe any significant changes in staurosporine-induced M-fusion protein reductions (Supplemental Fig. 5A and B).

We then examined Z-VAD-FMK, an inhibitor of cysteine proteases, including caspase, because staurosporine is an apoptosis inducer, namely, a caspase activator. Z-VAD-FMK inhibited staurosporine-induced reductions in M-fusion protein levels in all 3 *M-fusion* (+) ALL

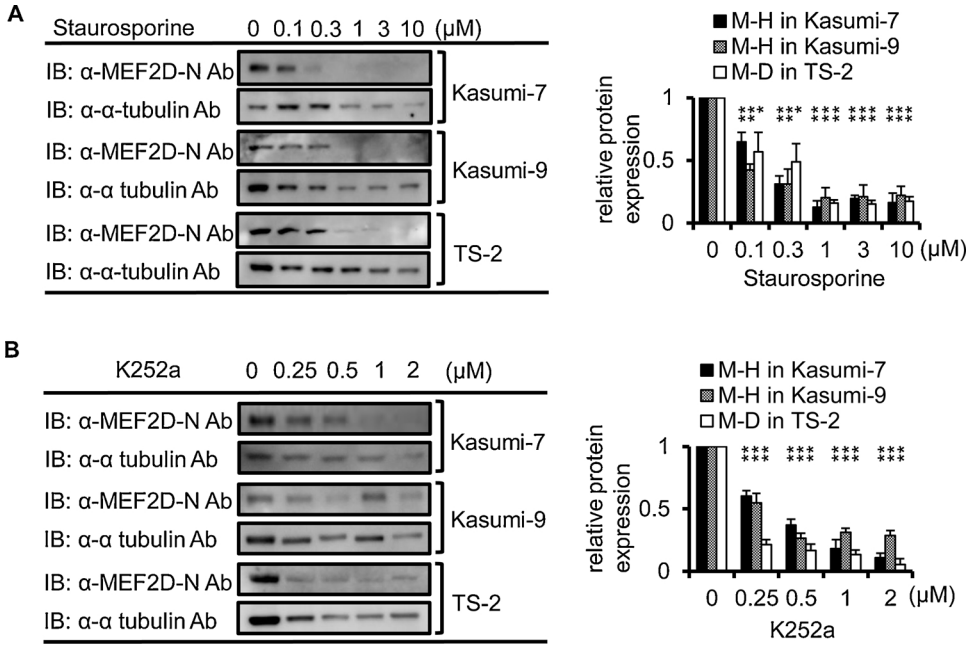


**Fig. 2.** Staurosporine and its analog reduced M-fusion protein expression in ALL cells. (A) Staurosporine reduced M-fusion protein expression in a dose-dependent manner. *M-fusion* (+) ALL cell lines were treated with staurosporine at the indicated concentration for 6 h and analyzed as in Fig. 1D. Differences in protein expression levels between untreated cells and cells treated with each concentration were statistically tested. **: $p < 0.01$, *: $p < 0.05$. (B) K252a had a weaker ability to induce the proteolysis of M-fusion proteins than staurosporine. *M-fusion* (+) ALL cell lines were treated with K252a at the indicated concentration for 6 h.

cell lines (Fig. 4A). Further analyses of Kasumi-7 confirmed the inhibited cleavage of caspase-3, caspase-7, and poly (ADP-ribose) polymerase (PARP) by Z-VAD-FMK (Fig. 4B). Z-VAD-FMK inhibits not only caspases, but also other cysteine proteases, such as cathepsin B, included in lysosomes, which are known to play an important role in autophagy; therefore, we examined the effects of caspase inhibition by a different mechanism to clarify the involvement of caspases in M-fusion protein reductions. iMAC1, a Bax channel blocker, successfully inhibited the staurosporine-induced cleavage of caspase-3, caspase-7, and PARP, and also inhibited M-fusion protein reductions (Fig. 4C). These results suggested that proteolysis by caspases was responsible for staurosporine-induced M-fusion protein reductions. We also confirmed that Z-VAD-FMK and iMAC1 both inhibited the staurosporine-induced apoptosis of *M-fusion* (+) ALL cells (Fig. 4D and E). Moreover, staurosporine-induced reductions in α-tubulin were inhibited by Z-VAD FMK (Fig. 4A), suggesting that they were mediated by caspase activation. A previous study reported that α-tubulin and β-actin are both substrates of caspase [19].

### 3.5. The cleavage at Asp125 by caspase was responsible for the staurosporine-induced proteolysis of M-fusion proteins in ALL cells

Caspases cleave peptides after Asp residues; however, many other requirements need to be met for Asp-containing peptides to become good caspase substrates. Although there are no definitive rules, the following have been proposed: A peptide with the sequence P4-P3-P2-Asp-P1, with Asp-P1 as a scissile bond, is a caspase substrate when 1) the P1 residue is a small and uncharged amino acid, such as Gly, Ser, or Ala; and 2) P4-P3-P2 residues are complementary for interactions with the catalytic groove of caspases [20]. To identify the caspase cleavage site of M-fusion proteins that was expected to exist in the MEF2D portion, we predicted cleavage sites using the web-based software cascleave [21] (Fig. 5A). We made expression vectors of the mutant M-H with the substitution of Asp in putative cleavage sites into Glu, and

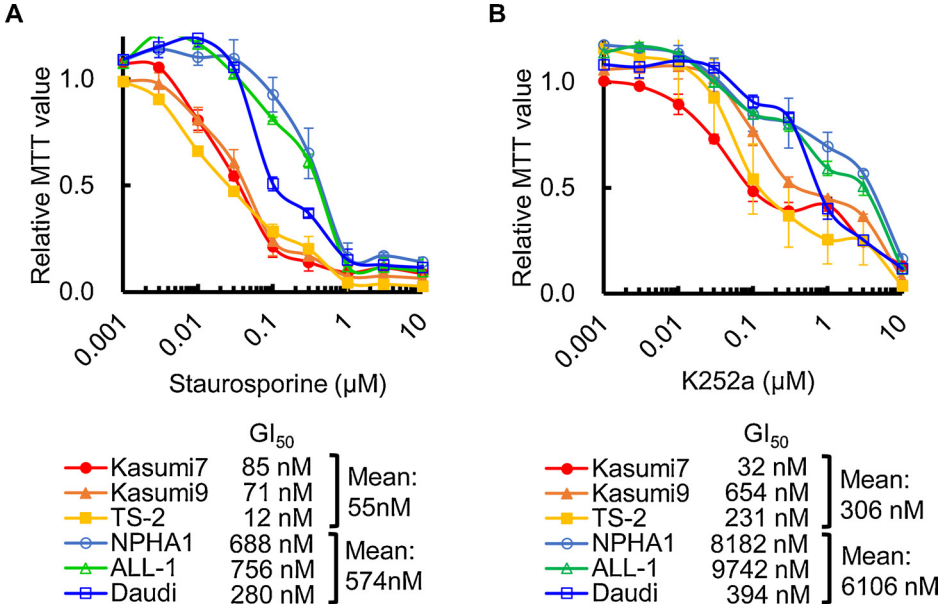


**Fig. 3.** Growth inhibitory effects of staurosporine and its analog were stronger in M-fusion (+) cell lines than in M-fusion (−) cell lines. (A) Dose-response curve of staurosporine. The indicated cell lines were treated with the indicated concentrations of staurosporine for 24 h. Viable cell numbers were analyzed using the MTT assay and presented as a relative value to control cells treated with DMSO. The mean value of at least 3 independent experiments is shown with error bars representing the standard deviation. (B) Dose-response curve of K252a. Analyses were performed as in (A).

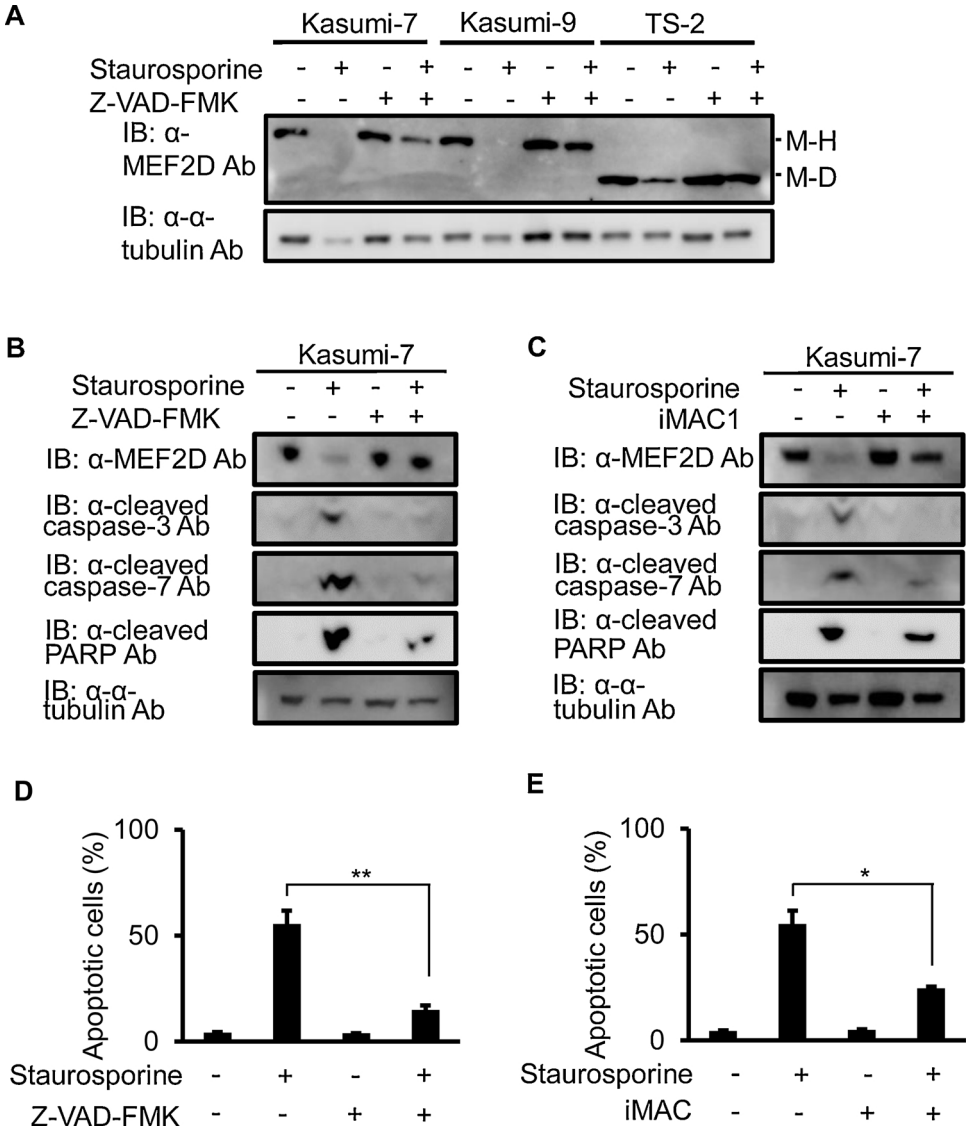


**Fig. 4.** Caspase inhibitors suppressed staurosporine-induced M-fusion protein reductions and apoptosis. (A) Z-VAD-FMK inhibited staurosporine-induced proteolysis. *M-fusion* (+) ALL cell lines were treated with 1 μM staurosporine and 50 μM Z-VAD-FMK as indicated for 6 h and analyzed as in Fig. 1D. (B) Staurosporine induced caspase activation. Kasumi-7 was treated as in (A) for 3 h, and analyzed as in Fig. 1D. (C) iMAC1 also inhibited staurosporine-induced caspase activation and the proteolysis of M-fusion proteins. Kasumi-7 was treated with 1 μM staurosporine and 5 μM iMAC1 for 3 h and analyzed as in Fig. 1D. (D)-(E) Z-VAD-FMK and iMAC1 inhibited staurosporine-induced apoptosis. Kasumi-7 was treated with 1 μM staurosporine and 50 μM Z-VAD-FMK (D) or 5 μM iMAC1 (E) for 3 h. Apoptosis was measured by staining with annexin-V and PI. Differences in the apoptotic cell ratio between the indicated samples were statistically tested. **: $p < 0.01$, *: $p < 0.05$.

performed the *in vitro* cleavage of wild-type and mutated M-H synthesized *via in vitro* translation/transcription. The substitution of Asp 125 into Glu (D125E) successfully abolished the cleavage of M-H by recombinant caspase-3 and caspase-7 (Fig. 5B), revealing that the cleavage site of M-H by caspases was Asp 125. Furthermore, the D125E mutation abolished the staurosporine-induced proteolysis of M-H in *M-fusion* (+) ALL cells, suggesting that the staurosporine-induced proteolysis of M-fusion proteins was due to cleavage by caspases at Asp 125 (Fig. 5C). In order to assess the role of the proteolysis of M-fusion proteins in staurosporine-induced apoptosis, we examined whether the introduction of the expression vector of M-H D125E into TS-2 caused resistance to staurosporine. However, the results obtained were inconclusive due to low cell viability of approximately 30 % after nucleofection and low gene introduction efficiency of approximately 30 % (data not shown). Therefore, we performed a detailed time-course experiment to investigate the temporal before-after relationship between caspase activation and the proteolysis of M-fusion proteins. The proteolysis of M-fusion proteins started at the same time as the activation of caspase-3, namely, 1 h after the staurosporine treatment, in all three *M-fusion* (+) ALL cell lines. As the proteolysis of M-fusion proteins proceeded, caspase activation was accelerated after approximately 4 h (Fig. 5D). These dynamics suggested the contribution of the proteolysis of M-fusion proteins to the progression of staurosporine-induced apoptosis.

### 3.6. *M-fusion* (+) ALL has potential as a novel target for venetoclax

Venetoclax is a B-cell lymphoma 2 (BCL-2) inhibitor that was recently approved by the FDA for CLL and AML in the elderly. The inhibition of BCL-2 by venetoclax induces apoptosis through the release of cytochrome c and activation of caspases [22], which is similar to staurosporine. Venetoclax-induced caspase activation may cause the proteolysis of M-fusion proteins, which may enhance the cytotoxic effects of venetoclax. In other words, *M-fusion* (+) ALL cells may be more sensitive to venetoclax than other cell lines. Therefore, we investigated whether venetoclax induced the proteolysis of M-fusion proteins and exerted stronger cytotoxic effects in *M-fusion* (+) ALL cells. As expected, venetoclax induced caspase-3 and caspase-7 activation and reductions in M-fusion protein levels in *M-fusion* (+) ALL cells; however, these effects were weaker in TS-2 cells (Fig. 6A). The growth inhibitory effects of venetoclax were stronger in *M-fusion* (+) ALL cells than in *M-fusion* (−) cells, and mean $GI_{50}$ were 26 and 130 nM, respectively (Fig. 6B). Consistent with the smaller M-fusion protein reductions observed in TS-2, they were also less sensitive to venetoclax. These results suggest *M-fusion* (+) ALL as a new target for venetoclax.

## 4. Discussion

The primary purpose of the present study was to establish a new

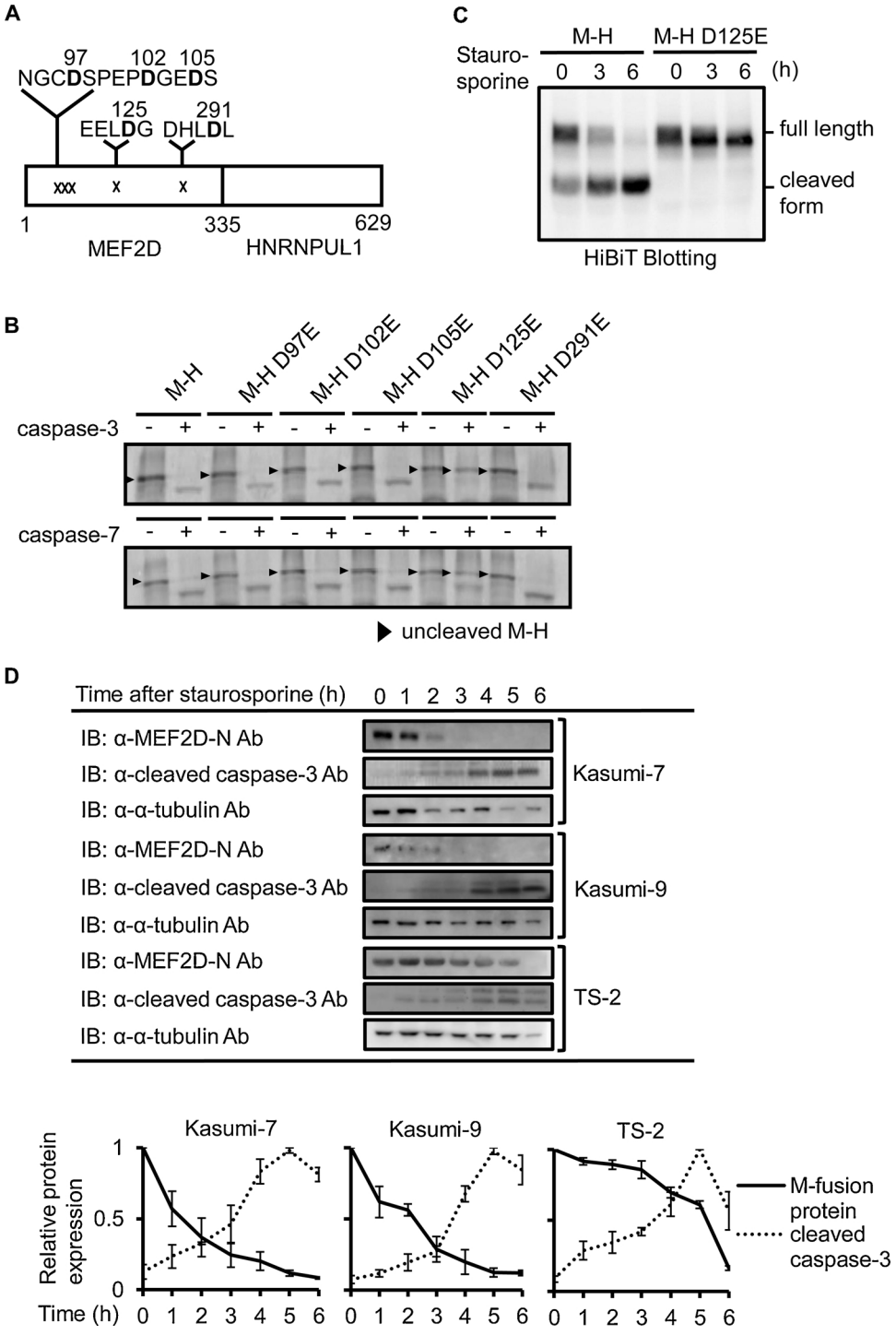


**Fig. 5.** Cleavage at Asp125 by caspase was responsible for the staurosporine-induced proteolysis of M-fusion proteins in ALL cells. (A) Schema of possible cleavage sites of M-H by caspase. Possible cleavage sites are presented in bold. (B) *In vitro* caspase cleavage assay. [$^{35}$S]-methionine-labeled M-H proteins with the indicated mutations were synthesized *in vitro*, incubated with recombinant caspase-3 and -7, resolved on SDS-PAGE, and visualized by autoradiography. The arrowheads indicate uncleaved M-H. The D125E mutation abolished the cleavage of M-H by caspase-3 and caspase-7 *in vitro*. (C) The D125E mutation abolished the staurosporine-induced proteolysis of M-H in ALL cells. The expression vectors of M-H and M-H D125E tagged with HiBiT peptides were introduced into TS-2, and treated with 1 μM staurosporine for the indicated time. The cleavage of M-H was detected by HiBiT blotting. (D) Reductions in M-fusion proteins started at the same time as the activation of caspase-3. *M-fusion* (+) ALL cell lines were treated with 1 μM staurosporine for the indicated time and analyzed as in Fig. 1D. The relative expression of M-fusion proteins and caspase-3 was potted on line charts.

high-throughput screening system that detects reductions in target proteins to make undruggable targets druggable. The coefficient variation value of the screening system that we developed was 4.7 % and the Z'-factor was 0.78 when we used staurosporine as a positive control, demonstrating the low measurement error and high accuracy of our system. Confirmation of the hit compounds in our system, staurosporine and K252a, inducing the proteolysis of M-fusion proteins provided a rationale for our screening system; therefore, the primary purpose was accomplished.

Non-enzymatic proteins, such as transcription factors, are recognized as undruggable targets. Several new technologies, such as proteolysis-targeting chimeric molecules (PROTACs), have been proposed to make undruggable targets druggable; however, it is also important to confirm that these targets are really undruggable. Since most cellular proteins are targets of degradation, autophagy, or proteolysis by proteases, it is possible to discover drugs that induce the proteolysis of undruggable targets by screening existing drugs and compound libraries using a high-throughput system that detects reductions in target proteins. Thalidomide, a drug that was originally developed as a sleeping aid and is now used to treat multiple myeloma, binds to cerebron, a substrate recognition component of the CUL4 ubiquitin ligase complex, and induces the degradation of IKZF1 and IKZF3 [23,24]. Similarly, indisulam, an anticancer sulfonamide, associates with DCAF15, a substrate recognition component of the CUL4

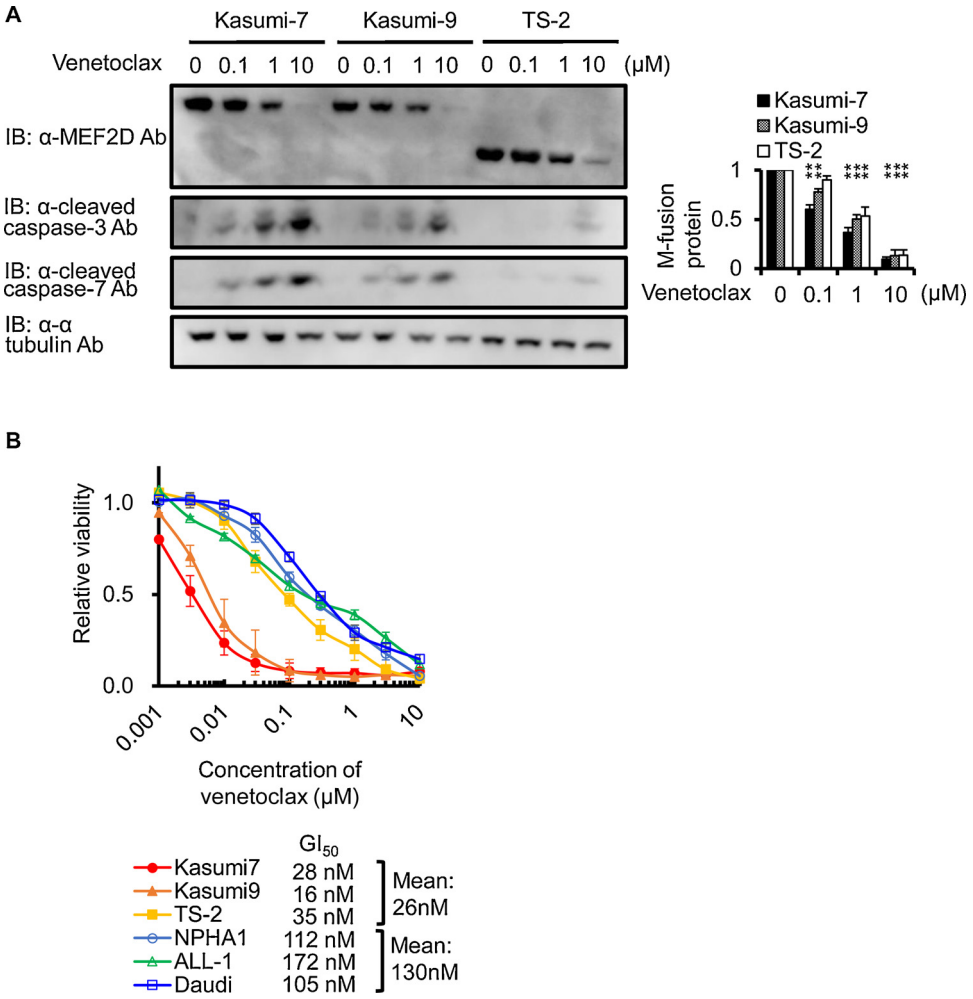


**Fig. 6.** Venetoclax, another caspase activator, induced the proteolysis of M-fusion proteins and inhibited the growth of *M-fusion* (+) ALL cells more strongly than *M-fusion* (−) cells. (A) Venetoclax induced caspase activation and the proteolysis of M-fusion proteins. *M-fusion* (+) ALL cell lines were treated with 1 μM venetoclax for the indicated time and analyzed as in Fig. 2A. (B) Dose-response curve of venetoclax. Analyses were performed as in Fig. 3A.

ubiquitin ligase complex, and induces the degradation of CAPERα [25,26]. These findings demonstrated that drugs that induce the proteolysis of undruggable targets may be discovered among existing drugs and compounds. It may be a reasonable strategy to set a target protein according to the pathology of a target disease and search for compounds inducing reductions in target proteins using a high-throughput screening system such as that developed herein.

Staurosporine is a multi-kinase inhibitor that is reported to have multiple mechanisms for the induction of apoptosis, such as caspase-dependent and independent mechanisms, and cytochrome c-dependent and independent caspase activation; however, the underlying molecular mechanisms have not yet been elucidated in detail [27,28]. It currently remains unclear whether the staurosporine-induced proteolysis of M-fusion proteins contributes to the apoptosis of *M-fusion* (+) ALL cells; however, we very recently demonstrated that the fusion gene-specific knockdown of M-H by short hairpin RNA caused apoptosis in Kasumi-7 [29]. This finding strongly indicates that M-fusion proteins confer survival advantages to *M-fusion* (+) ALL cells; therefore, the proteolysis of M-fusion proteins by caspase appears to contribute to the staurosporine-induced apoptosis of *M-fusion* (+) ALL cells. The proteolysis of M-fusion proteins and caspase activation may form a feed-forward loop to promote apoptosis, which increases the sensitivity of *M-fusion* (+) ALL cells to caspase activators, such as staurosporine and venetoclax.

The close analog of staurosporine, 7-hydroxy-staurosporine (UCN-01), was discovered in the 1980s, and many clinical trials have since been performed for leukemia, lymphoma, and other solid tumors. According to clinicalTrials.gov (https://clinicaltrials.gov), 16 phase I and 6 phase II studies have been conducted in the U.S., and all of them finished before 2013, except for one study with unknown findings; however, no phase III study has been initiated. Detailed reasons for the discontinuation of development have not been made public in most cases. One possible reason is its unexpectedly high affinity for the human serum protein, alpha-1 acid glycoprotein. The mean plasma concentration of UCN-01 at the recommended dose in the phase I study was 36.4 μM, whereas the mean salivary concentration was only 111 nM, which may lead to an insufficient clinical response to UCN-01 [30]. In addition, in the case of drugs with a high affinity to serum proteins, small changes in binding affinity by the disease status and the combination of drugs largely affect the free drug concentration, leading to large variations in drug efficiency and side effects. These disadvantages may have made the clinical use of staurosporine difficult; therefore, we searched for alternative drugs to staurosporine.

Based on the mechanism of cleavage of M-fusion proteins by caspase, we focused on another caspase activator, venetoclax. Venetoclax is a BH3-mimetic that inhibits anti-apoptotic proteins, such as BCL-2, BCL-w, and BCL-XL. It specifically binds to BCL-2 and prevents it from retaining proapoptotic proteins, such as BIM and BAX/BCL-2-associated death promoter (BAD), which leads to caspase activation [22]. If the above-described feed-forward loop between the proteolysis of M-fusion proteins and caspase activation exists, this loop will also function in venetoclax-induced apoptosis and increase the sensitivity of *M-fusion*

(+) ALL to venetoclax. In the present study, we observed the high sensitivity of *M-fusion* (+) ALL cell lines to venetoclax (Fig. 6B). Although venetoclax has been approved for the treatment of CLL and AML by the FDA, many clinical trials are being conducting to expand its indications to other hematological diseases, such as lymphoma and multiple myeloma [22]. The present results suggest that trials for *M-fusion* (+) ALL are warranted.

## 5. Conclusions

Through the establishment of a high-throughput and proteolysis-targeting screening system, we discovered staurosporine and venetoclax as inducers of the proteolysis of M-fusion proteins and demonstrated their potential as therapeutic agents for *M-fusion* (+) ALL. These results provide a rationale for our screening system and novel insights into drug screening strategies.

## Author contributions

F.H., N.T., and H.K. designed the study, performed experiments, and wrote the manuscript. T.Y., Y.M., D.H., T.S, S.Terakura., M.I., and S. Tsuzuki performed experiments. K.O. performed experiments for revision.

## Declaration of Competing Interest

H.K. received research funding from Astellas Pharma Inc., Bristol-Myers Squibb, Chugai Pharmaceutical Co., Ltd., Daiichi Sankyo Co., Ltd, Eisai Co., Ltd., FUJIFILM Corporation, Kyowa-Hakko Kirin Co., Ltd, Nippon Shinyaku Co., Ltd., Otsuka Pharmaceutical Co., Ltd., Perseus Proteomics Inc., Takeda Pharmaceutical Co., Ltd., Sumitomo Dainippon Parma Co., Ltd., and Zenyaku Kogyo Co., Ltd., and honoraria from Astellas Pharma Inc., Bristol-Myers Squibb, and Pfizer Japan Inc. The other authors have no potential conflicts of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.110330.

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