学位論文

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Abstract. *Background/Aim: Selective heat shock protein 90 (Hsp90) inhibitor SNX-2112 exhibits antitumor activity in multiple cancer cell types. Here, the antitumor activity of SNX-2112 in Nara-H cells was analyzed. Materials and Methods: Antitumor activity of SNX-2112 was assessed using a cell proliferation assay. We also examined the signalling pathways involved in SNX-2112-mediated autophagy and apoptosis of Nara-H cells by western blot and morphological analyses. Results: Cell proliferation assays demonstrated that SNX-2112 inhibited Nara-H cell growth. Western blotting revealed that SNX-2112 induced apoptosis and autophagy, inhibited mammalian target of rapamycin (mTOR) phosphorylation, and suppressed the mitogen-activated protein kinase (MAPK) signalling pathway. Morphological analysis confirmed that SNX-2112 induced autophagy and apoptosis. Conclusion: SNX-2112 induced autophagy and apoptosis of Nara-H cells by inhibiting mTOR and MAPK pathways. Our results support developing SNX-2112 to treat human soft tissue sarcomas.*

Undifferentiated pleomorphic sarcoma (UPS) is a heterogeneous tumour group without an identifiable lineage. Despite many chemotherapy protocols available for UPS, little consensus exists concerning appropriate doses and agent combinations (1, 2).

Heat shock proteins (Hsps) are potential targets for molecular targeted therapy. Cancer patients with high Hsp90 levels often have low survival rates. Thus, blocking Hsp90 may be a novel therapeutic strategy (3, 4), and the selective Hsp90 inhibitor SNX-2112 shows significant antitumor activity in several cancer cell types (5).

Autophagy has recently gained attention in regard to cancer pathogenesis and treatment, because of its paradoxical roles in cell survival and death (6). Hsp90 inhibition induces autophagy through suppression of the mammalian target of rapamycin (mTOR) (7). We previously demonstrated that an mTOR inhibitor induced cytoprotective autophagy in Nara-H cells, a high-grade UPS cell line (8). Autophagy protects cells against antitumor agents, and inhibiting autophagy enhances apoptosis (9-11). Here, the antitumor activity of SNX-2112 and the signalling pathways involved in SNX-2112-mediated autophagy and apoptosis were assessed in Nara-H cells.

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Key Words: SNX-2112, autophagy, apoptosis, undifferentiated pleomorphic sarcoma.

Materials and Methods

SNX-2112 and Nara-H cell culture. SNX-2112 was purchased from Selleckchem (Houston, TX, USA). Nara-H cells were obtained from ScienStuff (Nara, Japan) and cultured at 37°C with 5% CO_2.

Cell proliferation assay. The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to analyze cellular proliferation. Cells were seeded at 1×10^4 cells/well in 96-well plates. Forty-eight h later, fresh medium with 0, 0.4, 2, 10, or 50 μM SNX-2112 was added. After 24 or 48 h, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)2-(4-sulfophenyl)-2H-tetrazolium in 100 μl of fresh medium was added to each well. The cultures were incubated for 2 h, and then optical density was determined at 490 nm using an automatic microplate reader (Molecular Devices, Sunnyvale, CA, USA). At least three independent experiments were performed for each condition.

Western blotting. Cells were seeded at ~6×10^5 cells/well in six-well plates and cultured for 48 h. Cells were treated with SNX-2112 for 24 h and lysed in lysis buffer (Cell Signaling Technology, Beverly, MA, USA). Equivalent protein amounts were separated by SDS-polyacrylamide gel electrophoresis (Wako, Tokyo, Japan) under reducing conditions, electrophoretically transferred to nitrocellulose membranes (GE Healthcare Bio-Sciences, Piscataway, NJ, USA), and sequentially incubated with primary and secondary antibodies (Table I) in iBind solution (iBind Western System, Life Technologies, Carlsbad, CA, USA) for 2.5 h. Labelled proteins were visualized with a Novex® AP Chemiluminescent Detection Kit (Life Technologies) and LAS-1000 plus image analyzer (Fujifilm Co., Tokyo, Japan).

Immunocytochemistry of microtubule-associated protein light-chain 3 (LC3). Cells were plated at 1×10^6 cells/well on 25-mm round

Table I. Primary antibodies used in western blot analysis.

Target	Source	Host	Dilution	Secondary antibody	Gel (%)
HSP90	Cell Signaling	Rabbit	1:1,000	Anti-rabbit	10
Phospho-HSP90α	Cell Signaling	Rabbit	1:1,000	Anti-rabbit	10
4E-BP1	Cell Signaling	Rabbit	1:1,000	Anti-rabbit	10
Phospho-4E-BP1	Cell Signaling	Rabbit	1:1,000	Anti-rabbit	10
p70S6K	Cell Signaling	Rabbit	1:1,000	Anti-rabbit	10
Phospho-p70S6K	Cell Signaling	Rabbit	1:1,000	Anti-rabbit	10
MEK1/2	Cell Signaling	Rabbit	1:1,000	Anti-rabbit	10
Phospho-MEK1/2	Cell Signaling	Rabbit	1:1,000	Anti-rabbit	10
ERK1/2	Cell Signaling	Rabbit	1:1,000	Anti-rabbit	10
Phospho-ERK1/2	Chemicon	Rabbit	1:1,000	Anti-rabbit	10
B-Raf	Cell Signaling	Rabbit	1:1,000	Anti-rabbit	10
Cleaved Caspase-9	Cell Signaling	Rabbit	1:1,000	Anti-rabbit	12.5
Cleaved PARP	Cell Signaling	Rabbit	1:1,000	Anti-rabbit	10
α-tubulin	Cell Signaling	Rabbit	1:1,000	Anti-rabbit	10
Akt	Sigma	Rabbit	1:1,000	Anti-rabbit	10
Phospho-Akt	Sigma	Rabbit	1:1,000	Anti-rabbit	10
LC-3	MBL	Rabbit	1:1,000	Anti-rabbit	12.5
Atg5-Atg12 complex	MBL	Rabbit	1:1,000	Anti-rabbit	10
p62/SQSTM1	MBL	Rabbit	1:1,000	Anti-rabbit	10

coverslips (Matsunami Glass Ind. Ltd., Osaka, Japan) and cultured overnight. Cells were then treated with 25 μM SNX-2112 for 24 h, fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 30 min at room temperature, and then rinsed with PBS. To detect autophagy, fixed cells were stained with an anti-LC3 antibody (Code No. PM036; MBL, Nagoya, Japan) for 1 h at room temperature. After two washes, cells were incubated with an anti-IgG secondary antibody (Alexa Fluor® 488, Code No. A11008; Invitrogen, Carlsbad, CA, USA) for 30 min at room temperature and then viewed under an epifluorescence microscope (FSX100; Olympus Optical Co., Ltd., Tokyo, Japan).

Apoptosis detection by morphological assessment. Cells were plated and incubated with 25 μM SNX-2112 as described above. The cells were then stained with annexin V-FITC, propidium iodide (PI), and Hoechst 33342 for 15 min in the dark using reagents in the Promokine Apoptotic/Necrotic/Healthy Cells Detection Kit (PromoCell GmbH, Heidelberg, Germany).

Transmission electron microscopy. Cells were plated at 1×10^4 cells/well on a Lab-Tek® Chamber Slide (Nalge Nunc International, Naperville, IL, USA) and cultured for 48 h. After treatment with 25 μM of SNX-2112 for 24 h, the cells were rinsed, fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h, and post-fixed in 1% osmium tetroxide in the same buffer for 2 h. Ultrathin sections were stained with 4% uranyl acetate and lead citrate, and viewed under a JEM-1400 electron microscope (80 kV accelerating voltage; Jeol, Tokyo, Japan).

Statistical analysis. Proliferation assay results were assessed with one- or two-way analysis of variance, followed by *post-hoc* analysis. $p<0.05$ was considered as statistically significant. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA).

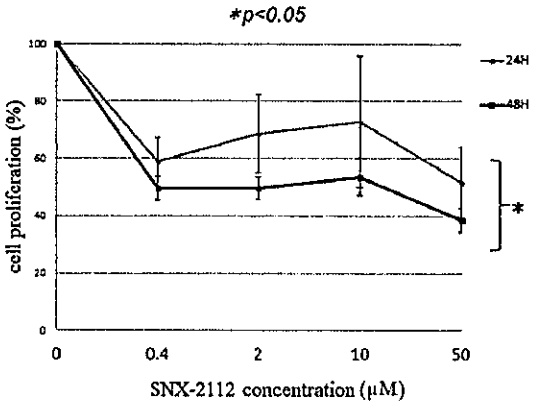

Figure 1. *SNX-2112 inhibits Nara-H cell proliferation.*

Results

SNX-2112 inhibits cell proliferation. SNX-2112 inhibited Nara-H cell proliferation in a dose and time-dependent manner (Figure 1).

Western blot analysis demonstrated that SNX-2112 induced apoptosis and autophagy, and inhibited mTOR and MAPK signalling pathways. HSP90 expression was high in Nara-H cells, and SNX-2112 decreased p-HSP90α expression in a dose-dependent manner (Figure 2A). SNX-2112 also induced dose and time-dependent down-regulation of p-p70S6K and p-4E-BP1, which are downstream of the Akt/mTOR

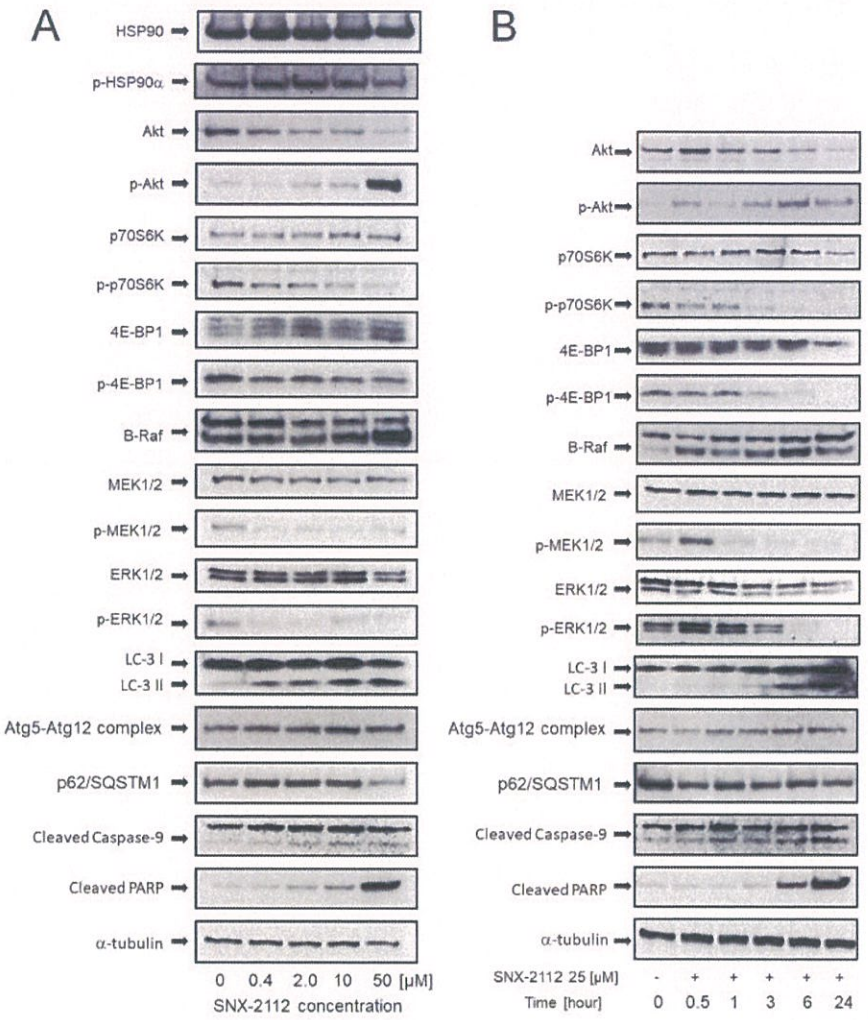

Figure 2. *Western blot analysis of SNX-2112 effects. (A) SNX-2112 induced dose-dependent down-regulation of mTOR and MAPK pathways, and apoptosis and autophagy were induced in Nara-H cells treated with SNX-2112. (B) Time-dependent effects of SNX-2112 in Nara-H cells.*

signalling pathway, and up-regulation of p-Akt (an activator of the Akt/mTOR signalling pathway) in Nara-H cells (Figure 2A and B).

In regard to B-Raf/mitogen-activated protein kinase (MAPK) signalling, treatment with SNX-2112 reduced p-MEK1/2 and p-ERK1/2 expression, while B-Raf expression was up-regulated in a dose and time-dependent manner (Figure 2A and B).

SNX-2112 treatment led to Poly (ADP-ribose) polymerase (PARP) cleavage and activation of caspase-9 in a dose and time-dependent manner in Nara-H cells, suggesting that SNX-2112 induced apoptosis (Figure 2A and B). Inhibition of PARP cleavage allows cells to maintain viability, whereas PARP cleavage facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (12).

SNX-2112 treatment increased expression of autophagy-related proteins LC3-II and Atg5-Atg12 complex. p62/SQSTM1 expression was decreased in a dose and time-dependent manner, indicating that SNX-2112 also induced autophagy of Nara-H cells (Figure 2A and B).

Morphological analysis revealed that SNX-2112 increased LC3-positive cells and apoptotic cells. LC3-positive cells were increased among SNX-2112-treated Nara-H cells compared with control cells (Figure 3A). In addition, in the presence of SNX-2112 annexin V-FITC-positive and annexin V-FITC/PI double-positive cells were identified, indicating apoptosis (Figure 3B).

Electron microscopy demonstrated that there were autophagic cells and apoptotic cells in SNX-2112 treated cells. Using

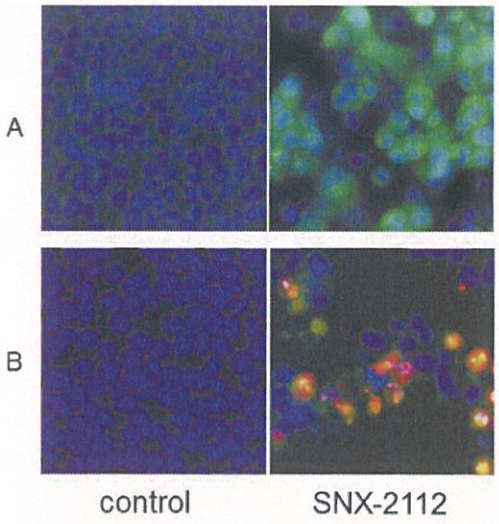

Figure 3. *Morphological analysis by fluorescence microscopy. (A) LC3 was used to evaluate autophagy. (B) Apoptotic cells were detected by annexin V, PI, and Hoechst 33342 triple staining.*

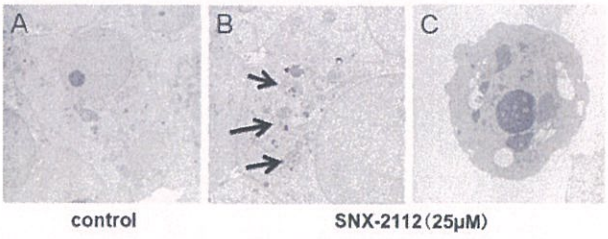

Figure 4. *Electron microscopy. (A) Control cell. (B) Autophagosomes (arrows). (C) Apoptotic cell.*

electron microscopy SNX-2112-treated cells were found to contain autophagosomes (Figure 4A and B). Apoptotic cells with nuclear fragmentation and chromatin condensation were also identified (Figure 4C).

Discussion

Molecular targeted drugs, including mTOR inhibitors, are being tested for sarcoma as monotherapy or in combination with other drugs such as autophagy inhibitors (13, 14). Because Hsp90 interacts with proteins in multiple signalling pathways necessary for cancer cell survival, such as mTOR and MAPK pathways, Hsp90 inhibitors have attracted great interest for cancer treatment. Hsp90 is constitutively expressed at 2-10-fold higher levels in tumour cells compared with normal cells, suggesting that Hsp90 may play a crucial role in the growth and survival of tumour cells (15). Moreover, Hsp90 interacting proteins induce apoptosis in various tumours (16). Therefore, Hsp90 inhibition is attractive as a therapeutic strategy for cancer.

Hsp90 blockage in cancer cells leads to protein breakdown and possibly lower proliferation. Clinical trials are testing Hsp90 inhibitors alone or in combination with other drugs as a therapy for multiple cancers (16). SNX-2112 inhibits, both *in vitro* and *in vivo*, growth of multiple cancer cell lines (17, 18). However, little is known regarding the activity of SNX-2112 in soft tissue tumours such as UPS. Thus, the antitumor effects of SNX-2112 were investigated in Nara-H cells. Consistent with observations in other cancers, SNX-2112 effectively inhibited Nara-H cell proliferation in a dose and time-dependent manner.

mTOR modulates several cell signalling events in the PI3K/Akt pathway that is important for the proliferation of many cancer cell types (14). mTOR interacts with other proteins and is the core component of two biochemically distinct complexes called mTORC1 and mTORC2. mTORC1, but not mTORC2, regulates autophagy and cell growth, the latter *via* p70S6K and 4E-BP1 phosphorylation (19, 20). We found LC3-positive cells, indicative of autophagy, following inhibition of Nara-H cell growth by Hsp90 blockage. This result suggests that SNX-2112 inhibited cell proliferation and induced autophagy by inhibition of the mTOR pathway in Nara-H cells.

Autophagy is involved in the routine turnover of cell constituents and regulated by mTOR and MAPK signalling pathways (20). The MAPK signalling pathway is critical for tumour formation (21). In this study, MAPK suppression and caspase-dependent apoptosis induction was observed, suggesting that SNX-2112 exerts its antitumor effects in part through apoptosis induced by inhibition of the MAPK pathway.

We demonstrated that SNX-2112 inhibited both mTOR and MAPK signalling pathways. Hsp90 interacts with multiple pathways, including mTOR and MAPK, through its association with relevant proteins, and inhibition of one pathway activates the other (22). Although the mechanism is not fully understood, some functional relationship appears to exist between apoptosis and autophagy (23-25). Our results indicated that suppression of the MAPK pathway induced apoptosis while suppression of the mTOR pathway induced autophagy. These data support the possibility of developing SNX-2112 to treat soft tissue sarcomas.

We previously showed that the Hsp90 inhibitor geldanamycin induced autophagy and apoptosis of osteosarcoma cells and an autophagy inhibitor enhanced apoptosis (26). Similarly, in chronic myeloid leukaemia, an autophagy inhibitor significantly enhanced apoptosis induced by Hsp90 inhibition (27). These reports suggested that autophagy control is important to improve antitumor effects of SNX-2112 because autophagy is a self-defence mechanism employed by cancer cells treated with antitumor agents (9, 11, 28). However, geldanamycin is too toxic for clinical use. Furthermore, SNX-2112 may suppress

autophagy induced by MAPK signalling and has a strong antitumor effect without the need to be combined with an autophagy inhibitor. Therefore, SNX-2112 was used in this study.

In conclusion, autophagy and apoptosis were induced in Nara-H cells treated with SNX-2112. Inhibition of phosphorylation of signalling molecules in mTOR and MAPK pathways played a role in these events. Our data support the development of SNX-2112 as a treatment for human soft tissue sarcoma.

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Nomenclature and Abbreviations. Nomenclature should follow that given in "Chemical Abstracts", "Index Medicus", "Merck Index", "IUPAC -IUB", "Bergey's Manual of Determinative Bacteriology", The CBE Manual for Authors, Editors and Publishers (6th edition, 1994), and MIAME Standard for Microarray Data. Human gene symbols may be obtained from the HUGO Gene Nomenclature Committee (HGNC) (http://www.gene.ucl.ac.uk/). Approved mouse nomenclature may be obtained from http://www.informatics.jax.org/. Standard abbreviations are preferable. If a new abbreviation is used, it must be defined on first usage.

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