Deficiency of Fyn protein is prerequisite for apoptosis induced by Src family kinase inhibitors in human mesothelioma cells

Ryoi Eguchi1,2, Shuji Kubo3, Hiromi Takeda1, Toshiro Ohita4, Chiharu Tabata2, Hiroyasu Ogawa1, Takashi Nakano2 and Yoshihiro Fujimori1,4

1Laboratory of Cell Transplantation, Institute for Advanced Medical Sciences, 2Department of Thoracic Oncology and 3Department of Genetics, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan and 4Department of Food and Nutritional Sciences, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka 422-8526, Japan

To whom correspondence should be addressed. Tel/Fax: +81 798 45 6599; Email: fuji-y@hyo-med.ac.jp

Malignant mesothelioma is an aggressive tumor arising from mesothelial cells of serous membranes. Src family kinases (SKFs) have a pivotal role in cell adhesion, proliferation, survival and apoptosis. Here, we examined the effect of SKF inhibitors in NCI-H2052, ACC-MESO-4 and NCI-H28 cells, mesothelioma cell lines and Met5A, a human non-malignant mesothelial cell line. We found that PP2, a selective SKF inhibitor, inhibited SKF activity and induced apoptosis mediated by caspase-8 in NCI-H28 but not Met5A, NCI-H2052 and ACC-MESO-4 cells. Src, Yes, Fyn and Lyn protein, which are members of the SKF, were expressed in these cell lines, whereas NCI-H28 cells were deficient in Fyn protein. Small interfering RNA (siRNA) targeting Fyn facilitated PP2-induced apoptosis mediated by caspase-8 in NCI-H2052 and ACC-MESO-4 cells. PP2 reduced Lyn protein levels and suppressed SKF activity in all mesothelioma cell lines. Lyn siRNA induced caspase-8 activation and apoptosis in NCI-H28 cells but not in NCI-H2052 and ACC-MESO-4 cells. However, double RNA interference knockdown of Fyn and Lyn induced apoptosis accompanied by caspase-8 activation in NCI-H2052 and ACC-MESO-4 cells. Dasatinib, an inhibitor of multi-tyrosine kinases including SFK, also inhibited SKF activity and induced reduction of Lyn protein levels, caspase-8 activation and apoptosis in NCI-H28 cells but not in other cell lines. Present study suggests that SKF inhibitors induce caspase-8-dependent apoptosis caused by reduction of Lyn protein in Fyn-deficient mesothelioma cells.

Introduction

Malignant mesothelioma is an aggressive tumor arising from mesothelial cells of serous membranes, including pleura, peritoneum and pericardium (1–3). Mesothelioma is highly resistant to most chemotherapeutic agents (3), and radiation and surgical therapy generally show limited efficacy (3–5). New approaches for the treatment of malignant mesothelioma are therefore required. Src family kinases (SKFs) are non-receptor and cytoplasmic tyrosine kinases that have a critical role in cell adhesion, proliferation, survival and apoptosis. In SSKs, Src, Yes and Fyn show ubiquitous expression, whereas others, including Lyn, exhibit more restricted tissue localization (6,7). SKF binds to focal adhesion kinase (FAK), a widely expressed cytoplasmic protein tyrosine kinase. Within FSK–FAK complex, SFK can trans-phosphorylate Tyr-576 and Tyr-577 in the kinase domain of FAK, which regulates migration, cell spreading and focal contact during cell motility (8,9).

In response to apoptotic stimuli, caspases relay messages through so-called initiator caspases to effector caspases (10). Dephosphorylation of Tyr-380, Tyr-397 and Tyr-465 in caspase-8 activation (11,12). Caspase cascade mediates apoptotic processes, such as externalization of phosphatidylserine, followed by cell death (10).

To explore a new therapeutic target against mesothelioma, we investigated the effects of SKF inhibitors, PP2 and dasatinib, on human mesothelioma cell lines, NCI-H2052, ACC-MESO-4 and NCI-H28 cells and a human non-malignant mesothelial cell line, Met5A. We found that SKF inhibitors induced apoptosis in NCI-H28 cells, which were deficient in Fyn protein. Further clarification of cell signaling pathways revealed that deficiency of Fyn protein is prerequisite for apoptosis induced by SKF inhibitors in human mesothelioma cells.

Materials and methods

Cell lines and culture

A non-malignant transformed human pleural mesothelial cell line, Met5A and two human mesothelioma cell lines, NCI-H2052 and NCI-H28, were obtained from the American Type Culture Collection (Rockville, MD). Another human mesothelioma cell line, ACC-MESO-4, was purchased from the RIKEN BioResource Center (Tsukuba, Japan). Cells were cultured as monolayers in RPMI-1640 medium (Sigma, St Louis, MO) with 10% fetal bovine serum (Sigma) at 37°C under a humidified atmosphere containing 5% CO2.

Reagents and inhibitors

Antitumor agents and inhibitors were prepared in dimethyl sulfoxide using the following stock solutions: 10 mM PP2, 20 mM zEt(OMe)/TD(OMe)-fmk, 40 mM zVAD(OMe)-fmk (BIOMOL, Plymouth Meeting, PA), 25 mM MG132 (BIOMOL) and 5 mM dasatinib (Biovision, Mountain View, CA). Dimethyl sulfoxide was used as a vehicle control as appropriate. All other chemicals were purchased from Sigma.

Treatment with antitumor agents and inhibitors

Cells were seeded as described below and cultured for 24 h. Old medium was aspirated, and fresh medium containing PP2 or dasatinib was added. For PP2 treatment, zVAD, MG132 or vehicle (dimethyl sulfoxide) was added to fresh medium containing PP2. Cells were then cultured for 3–72 h.

Cell viability analysis

Cells were seeded in a 96-well plate (Becton Dickinson Labware, Franklin Lakes, NJ) at 2 × 104 cells per well. Cell proliferation was determined by a colorimetric assay using Cell Counting Kit-8 (Dojin Chemical Institute, Kumamoto, Japan) according to the manufacturer’s protocol. Color intensity was quantified as described earlier (13).

Western blotting and antibodies

Western blotting was performed as described earlier (13). Antibodies used to detect phospho-Src family (Tyr-416, #2101), phospho-FAK (Tyr-576/577, #3281), full-length and cleaved caspase-8 (#9746), caspase-3 (#9662), cleaved caspase-3 (#9661), Src (#2108) and phospho-Lyn (Tyr-507, #2731) were purchased from Cell Signaling Technology (Beverly, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (#F-316) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). FAK (#610087), Yes (#610375), Fyn (#610163) and Lyn antibody (#610003) were purchased from BD Biosciences (San Jose, CA). All western blot analyses were performed three times and the representative data are shown.

Flow cytometric analysis of apoptosis

Apoptosis was analyzed by flow cytometry using an Annexin V (Ax)-fluorescein isothiocyanate Kit (Medical & Biological Laboratories Co. Ltd, Nagoya, Japan) as described earlier (13). Briefly, 1 × 105 cells in a 60 mm dish (Becton Dickinson Labware) treated with PP2 or dasatinib were trypsinized, washed with phosphate-buffered saline and then labeled with Ax-fluorescein isothiocyanate and propidium iodide. Fluorescence intensity was measured using a Cytomics FC 500 flow cytometer and CXP software (Beckman Coulter, Fullerton, CA).

Quantitative reverse transcription–PCR analysis

Total RNA was isolated from cells using TRIzol reagent (Enzo Life Sciences, Farmingdale, NY). First-strand complementary DNA was synthesized from the
total RNA (1.25 μg) using the PrimeScript RT reagent kit (Takara Bio, Ohtsu, Japan) according to the manufacturer’s instructions. PCR was performed on the synthesized complementary DNA product using TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. All reactions were carried out in triplicate. The sequences of the PCR primer pairs and fluorogenic probes used for Fyn and GAPDH are available on the Applied Biosystems website (http://www.appliedbiosystems.com/absite/us/en/home.html); Fyn assay ID: Hs00176628_m1; GAPDH assay ID: Hs99999905_m1). The amplification conditions were as follows: denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min and 40 cycles. The PCR products were analyzed using the ABI 7500 real-time PCR system (Applied Biosystems). Each messenger RNA (mRNA) level was normalized to the corresponding GAPDH mRNA level used as an internal control.

RNA interference
Small interfering RNAs (siRNAs) targeting Fyn [FYN Stealth Select RNA interference (RNAi) HSS103882], Yes (YES Stealth Select RNAi HSS103883) and Lyn (LYN Stealth Select RNAi HSS106213) and Stealth RNAi Negative Control Duplexes were purchased from Invitrogen (Carlsbad, CA). As control siRNA, #12935-146 (#146), #12935-115 (#115) and #12935-147 were used in NCI-H2052, ACC-MESO-4 and NCI-H28 cells, respectively. Stealth siRNA duplex oligoribonucleotides against Src (GenBankTM accession number NM_005417) were synthesized by Invitrogen. The sequences were as follows: sense 5′-CUGUGUUGUGACAAUCUGGAGCCG-3′ and antisense 5′-CGGCCUCCAGAUGUCAAACAACAG-3′. The duplex oligoribonucleotides were dissolved in diethyl pyrocarbonate-treated water to make 500 nM. Transient transfection of siRNAs was carried out using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Stealth RNAi compounds were used at a concentration of 10 nM in transfection as described earlier (13). Briefly, 2 × 10⁵ cells were incubated in 10 ml of RPMI-1640 medium in a 100 mm dish overnight. Lipofectamine RNAiMAX and siRNA were each dissolved in 1 ml RPMI-1640 medium for 5 min at room temperature, combined and incubated for 15 min at room temperature to form complexes. Two milliliters of the mixture was added to the cell culture and incubated for 48 h. The cells were harvested by trypsinization and were seeded in a dish for PP2 treatment as described above.

Statistical analysis
All data are presented as the mean ± standard error of three independent experiments. Comparisons between two groups were performed using Student’s unpaired t-test (* P < 0.05, **P < 0.01 and ***P < 0.005).

Results
PP2 induces apoptosis mediated by caspase-8 in NCI-H28 cells
We analyzed the effects of PP2, a selective SFK inhibitor, on mesothelioma cell lines (Figure 1). PP2 suppressed cell viability of all mesothelioma cell lines used more markedly than that of Met5A cells in a concentration-dependent manner (Figure 1A). PP2 suppressed cell viability of all mesothelioma cell lines used more markedly than that of Met5A cells in a concentration-dependent manner (Figure 1A). We used an antibody recognizing phosphorylation of tyrosine residue corresponding to Tyr-416 of Src in various SFKs (Figure 1B). PP2 inhibited the phosphorylation of SFK (lower two arrows in Figure 1B) and that of p-FAK in a concentration-dependent manner in all mesothelioma cell lines used. These results show that enzyme activity of SFK is suppressed by PP2. Intriguingly, PP2 significantly induced apoptosis in NCI-H28 cells (Figure 1C). In NCI-H28 cells, PP2 also induced cleavage of caspase-8 and caspase-3 in a time-dependent manner (Figure 1D). Furthermore, PP2-induced apoptosis in NCI-H28 cells

Fig. 1. PP2 induces apoptosis mediated by caspase-8 in NCI-H28 cells. (A) PP2 suppresses cell viability of NCI-H2052, ACC-MESO-4 and NCI-H28 cells more markedly than that of Met5A cells. These cell lines were treated with PP2 for 48 h at the indicated concentrations. Cell viability was assessed using the Cell Counting Kit-8 assay. (B) PP2 suppresses enzyme activity of SFK. Cell extracts were prepared from NCI-H2052, ACC-MESO-4 and NCI-H28 cells treated with 5–20 μM PP2 for 6 h as described in Materials and methods. Arrows indicate phosphorylation of Tyr-416 in various SFKs. (C) PP2 induces apoptosis in NCI-H28 cells, but not in Met5A, NCI-H2052 and ACC-MESO-4 cells. The numbers of (Ax (+)) apoptotic cells were markedly increased 72 h after the treatment with 20 μM PP2 in NCI-H28 cells but not in other cell lines. (D) PP2 induces activation of caspase-8 and caspase-3. Cell extracts were prepared from NCI-H28 cells treated with 20 μM PP2 for 12–48 h. (E) PP2-induced apoptosis in NCI-H28 cells is suppressed by zV AD-fmk and zIETD-fmk. NCI-H28 cells were treated with 20 μM PP2 alone or together with 100 μM zVAD-fmk and 50 μM zIETD-fmk for 72 h and analyzed for Ax (+) apoptotic cells by flow cytometry.
was significantly suppressed by zVAD-fmk, a broad-spectrum caspase inhibitor, and zIETD-fmk, a specific caspase-8 inhibitor (Figure 1E). These results suggest that PP2 suppresses SFK activity and induces apoptosis mediated by caspase-8 in NCI-H28 cells.

**Deficiency of Fyn protein is caused by transcriptional repression of Fyn mRNA in NCI-H28 cells, and Lyn is expressed in mesothelial and mesothelioma cells**

We examined whether SFK expression is involved in PP2-induced apoptosis in mesothelioma cells (Figure 2). Src, Yes and Lyn protein were detected in all cell lines used, but NCI-H28 cells were deficient in Fyn protein, whereas it was expressed in other three cell lines (Figure 2A). Phosphorylation of SFK in NCI-H2052 and ACC-MESO-4 but not NCI-H28 cells was higher than that in Met5A cells. We then examined expression of Fyn mRNA using quantitative reverse transcription–PCR (Figure 2B). It was found that Fyn mRNA expression in NCI-H28 cells was much lower than that in other cell lines used. Additional experiments using other PCR primer pairs and fluorogenic probes also showed that Fyn mRNA was not completely absent in NCI-H28 cells (data not shown). These results suggest that deficiency of Fyn protein is caused by transcriptional repression of Fyn mRNA in NCI-H28 cells and that Lyn is expressed in mesothelial and mesothelioma cells.

**Fyn-knockdown facilitates PP2-induced apoptosis in mesothelioma cells**

To further investigate whether transcriptional repression of Fyn mRNA is related to PP2-induced apoptosis, we performed RNAi using siRNA targeting Fyn in NCI-H2052 and ACC-MESO-4 cells (Figure 3). Fyn siRNA abrogated expression of Fyn protein with no effect on expression of Src, Yes and Lyn protein (Figure 3A). In NCI-H2052 and ACC-MESO-4 cells treated with Fyn siRNA, PP2 induced apoptosis more significantly than in these cells treated with control siRNA (Figure 3B). Furthermore, PP2 induced cleavage of caspase-8 and caspase-3 in these Fyn-knockdown mesothelioma cell lines (Figure 3C). These results suggest that Fyn-knockdown facilitates PP2-induced apoptosis in Fyn-expressing mesothelioma cells.

**SFK inhibition induces apoptosis in Fyn-deficient mesothelioma cells**

We then analyzed whether PP2-induced apoptosis is related to SFK inhibition in NCI-H28 cells (Figure 4). Western blot analysis showed that PP2 suppressed phosphorylation of Tyr-416 in SFKs and that of Fig. 2. Deficiency of Fyn protein is caused by transcriptional repression of Fyn mRNA in NCI-H28 cells. (A) Expression and phosphorylation of SFK in Met5A, NCI-H2052 and ACC-MESO-4 and NCI-H28 cells. Cell extracts were prepared from these cell lines incubated for 72 h. (B) Fyn synthesis is suppressed by transcriptional repression of Fyn mRNA in NCI-H28 cells. Total RNA was isolated from these cells after incubation for 72 h as described in Materials and methods.

Fig. 3. Fyn-knockdown facilitates PP2-induced apoptosis in mesothelioma cells. (A) Fyn-knockdown in mesothelioma cells. NCI-H2052 and ACC-MESO-4 cells were transfected with 10 nM siRNA against Fyn mRNA or 10 nM control siRNA for 48 h, harvested by trypsinization and incubated for 24 h. Cell extracts were prepared from NCI-H2052 and ACC-MESO-4 cells after the incubation for 24 h. (B) Fyn-knockdown facilitates PP2-induced apoptosis in mesothelioma cells. NCI-H2052 and ACC-MESO-4 cells, transfected with Fyn and control siRNAs, were treated with 20 μM PP2 for 72 h and analyzed for Ax (+) apoptotic cells by flow cytometry. (C) PP2 induces activation of caspase-8 and caspase-3 in Fyn-knockdown mesothelioma cells. NCI-H2052 and ACC-MESO-4 cells, transfected with Fyn and control siRNA, were treated with 20 μM PP2 for 48 h.
Tyr-507 in Lyn (Figure 4A). PP2 also induced reduction of Lyn protein levels but not Src or Yes protein levels. These results suggest that PP2-inhibited Lyn phosphorylation is caused by reduction of Lyn protein. Lyn protein has been reported to be degraded by the ubiquitin-proteasome pathway (14). Therefore, we examined the involvement of this pathway. MG132, a proteasome inhibitor, suppressed PP2-induced reduction of Lyn protein (Figure 4B), suggesting that Lyn protein is degraded by the ubiquitin-proteasome pathway. To investigate which SFKs are involved in apoptosis mediated by caspase-8 in Fyn-deficient mesothelioma cells, we analyzed the induction of apoptosis in NCI-H28 cells treated with siRNAs targeting Src, Yes and Lyn. Each siRNA abrogated expression of respective SFK protein (Figure 4C). Src and Lyn siRNAs, but not Yes siRNAs, significantly induced apoptosis in NCI-H28 cells (Figure 4D). In addition, knockdown of Src and Lyn, but not of Yes, induced cleavage of caspase-8 and caspase-3. These results suggest that Src and Lyn suppress caspase-8 activation and apoptosis in Fyn-deficient mesothelioma cells.

Both Fyn and Lyn suppress apoptosis in mesothelioma cells
We analyzed whether Src, Yes and Lyn are involved in apoptosis in Fyn-expressing mesothelioma cells (Figure 5). In NCI-H2052 and ACC-MESO-4 cells treated with Fyn siRNA and control siRNA, PP2 suppressed FAK phosphorylation and reduced Lyn protein levels (Figure 5A), whereas PP2 failed to induce caspase-8 cleavage and apoptosis in these cells treated with control siRNA (Figure 5B). We then investigated apoptotic induction by RNAi knockdown of Src, Yes and Lyn in Fyn-expressing mesothelioma cells. Individual siRNAs abrogated expression of respective SFK protein but failed to induce apoptosis in NCI-H2052 and ACC-MESO-4 cells (Figure 5C). We further performed knockdown of both Fyn and Lyn. The double knockdown of Fyn and Lyn efficiently abrogated expression of both Fyn and Lyn with no effect on expression of Src and Yes protein (Figure 5D). The double knockdown induced significant apoptosis and cleavage of caspase-8 and caspase-3 in NCI-H2052 and ACC-MESO-4 cells (Figure 5D). Collectively, these results show that PP2-induced apoptosis is caused by reduction of Lyn protein in Fyn-deficient mesothelioma cells.

SFK inhibitors induce reduction of Lyn protein followed by caspase-8-dependent apoptosis in Fyn-deficient mesothelioma cells
To further investigate whether other SFK inhibitors are able to induce apoptosis in Fyn-deficient mesothelioma cells, we treated these cell lines with dasatinib, an inhibitor of multi-tyrosine kinase including Bcr-Abl kinase and SFK (Figure 6). Dasatinib suppressed cell viability of all MM cell lines used more markedly than that of Met5A cells in a concentration-dependent manner (Figure 6A). In addition, Dasatinib significantly induced apoptosis in NCI-H28 cells but not other mesothelioma cell lines (Figure 6B). Dasatinib inhibited phosphorylation of SFKs and FAK, reduced Lyn protein, but not of Src and Yes protein levels and cleavage of caspase-8 and caspase-3 in a concentration- and time-dependent manner in NCI-H28 cells (Figure 6C). Dasatinib also induced reduction of Lyn protein levels in NCI-H2052 and ACC-MESO-4 cells (data not shown) but failed to induce cell death in these Fyn-expressing mesothelioma cell lines.
These results suggest that dasatinib, like PP2, is able to induce caspase-8-dependent apoptosis mediated by reduction of Lyn protein in Fyn-deficient mesothelioma cells.

**Discussion**

Malignant mesothelioma is refractory to conventional chemotherapy, which is related to the resistance to induction of apoptosis by chemotherapeutic agents (3). PP2 has been shown to induce apoptosis in some tumor cells, including B-cell leukemia and breast cancer and hepatoma but not in primary hepatocytes (15–17). In the present study, we found that PP2 induced apoptosis mediated by caspase-8 in a mesothelioma cell line, NCI-H2052, but not a non-malignant mesothelial cell line, Met5A (Figure 1).

Fyn is generally expressed in various tissues (7). Fyn has been recognized as an important mediator of cell cycle, growth, survival and cell–cell adhesion (18). Western blot and quantitative reverse transcription–PCR analyses revealed that Fyn protein is detected in all cell lines used except NCI-H28 cells, and Fyn synthesis is suppressed by transcriptional repression of Fyn mRNA in NCI-H28 cells (Figure 2). In prostate cancer, Fyn is reported to be downregulated by both chromosomal deletion and promoter hypermethylation (19). However, Fyn promoter regions were not methylated in NCI-H28 cells (data not shown). Detailed investigation to clarify how transcription of Fyn mRNA is repressed in NCI-H28 cells is needed. We also found that Fyn-knockdown facilitated PP2-induced apoptosis in mesothelioma cells (Figures 3 and 5). Fyn is overexpressed in various cancers, including glioblastoma, squamous cell carcinoma, melanoma and chronic myeloid leukemia (18,20). In addition, chronic myeloid leukemia cells overexpressing Fyn have been shown to be resistant to PD166326, a Bcr-Abl kinase and SFK inhibitor (21). We found that Fyn-knockdown or deficiency of Fyn protein is insufficient to induce apoptosis but facilitated PP2-induced apoptosis in mesothelioma cells (Figures 1–3).

Lyn is known to be expressed in all blood cells except T lymphocytes (22). In neutrophils, PP2 is reported to reduce Lyn protein levels and apoptosis accompanied by caspase-8 activation (12). Our results revealed that Lyn is expressed in mesothelial and mesothelioma cells and degraded by the ubiquitin-proteasome pathway in mesothelioma cells (Figures 2 and 4). Lyn is overexpressed in various cancers, including glioblastoma, squamous cell carcinoma, melanoma and chronic myeloid leukemia (18,20). In addition, chronic myeloid leukemia cells overexpressing Lyn have been shown to be resistant to PP2-induced apoptosis (21). We found that Lyn-knockdown or deficiency of Lyn protein is insufficient to induce apoptosis but facilitated PP2-induced apoptosis in mesothelioma cells (Figures 1–3).

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and Lyn-knockdown failed to induce apoptosis in other mesothelioma cell lines expressing Fyn (Figures 2, 3 and 5). Intriguingly, double knockdown of Fyn and Lyn induced caspase-8 activation and apoptosis in the mesothelioma cell lines expressing Fyn (Figure 5). These results suggest that Lyn plays a pivotal role in apoptosis of Fyn-deficient mesothelioma cells. We need to further investigate how Lyn and Fyn regulate apoptosis of mesothelioma cells.

Dasatinib is a highly potent Bcr-Abl kinase inhibitor used for the treatment of imatinib-resistant chronic myeloid leukemia (23). Dasatinib also inhibits SFK activity, leading to induction of apoptosis and cell cycle arrest and suppression of cell migration and invasion in mesothelioma cells (24). However, whether the apoptotic mechanism of dasatinib is the same as that of PP2 was not clear. We found that dasatinib inhibited SFK activity, reduced Lyn protein and induced apoptosis mediated by caspase-8 in NCI-H28 cells in a similar manner as PP2 (Figure 6). Recently, it has been reported that Src and Fyn are relevant targets for dasatinib action in lung cancer and that Lyn is a mediator of epithelial–mesenchymal transition and a target of dasatinib in breast cancer (25,26). These results suggest that dasatinib may be an effective molecular target drug against Fyn-deficient mesothelioma cells.

Preferential susceptibility to kinase inhibitor in NCI-H28 cells is also reported in c-Met, a receptor tyrosine kinase of hepatocyte growth factor, which is involved in cell growth, survival and migration (27). It has been shown that c-Met is overexpressed in NCI-H28 cells and that SU11274, a c-Met inhibitor, suppresses cell growth in NCI-H28 cells. If SU11274 induces reduction of Lyn protein in NCI-H28 cells, c-Met inhibitors as well as SFK inhibitors may induce apoptosis in Fyn-deficient mesothelioma cells.

In conclusion, SFK inhibitors, PP2 and dasatinib, reduce Lyn protein, leading to apoptosis mediated by caspase-8 in Fyn-deficient mesothelioma cells (Figure 6D). This study also shows potential utility of SFK inhibitors in the treatment of malignant mesothelioma and for the first time that deficiency of Fyn protein is prerequisite for apoptosis induced by SFK inhibitors in mesothelioma cells. Deficiency of Fyn protein would be a biomarker that discriminate the
sensitivity of SFK inhibitors in molecular-targeted therapy to mesothelioma. Additionally, further studies may open a novel way to develop therapy with both Fyn-deficiency and Lyn inhibition for this malignancy and other human tumor.

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**References**


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