Glycogen synthase kinase 3β as a potential therapeutic target in synovial sarcoma and fibrosarcoma

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Abstract
Soft tissue sarcomas (STSs) are a rare cancer type. Almost half are unresponsive to multi-pronged treatment and might therefore benefit from biologically targeted therapy. An emerging target is glycogen synthase kinase (GSK3β), which is implicated in various diseases including cancer. Here, we investigated the expression, activity and putative pathological role of GSK3β in synovial sarcoma and fibrosarcoma, comprising the majority of STS that are encountered in orthopedics. Expression of the active form of GSK3β (tyrosine 216-phosphorylated) was higher in synovial sarcoma (SYO-1, HS-SY-II, SW982) and in fibrosarcoma (HT1080) tumor cell lines than in untransformed fibroblast (NHDF) cells that are assumed to be the normal mesenchymal counterpart cells. Inhibition of GSK3β activity by pharmacological agents (AR-A014418, SB-216763) or of its expression by RNA interference suppressed the proliferation of sarcoma cells and their invasion of collagen gel, as well as inducing their apoptosis. These effects were associated with G0/G1-phase cell cycle arrest and decreased expression of cyclin D1, cyclin-dependent kinase (CDK)4 and matrix metalloproteinase 2. Intraperitoneal injection of the GSK3β inhibitors attenuated the growth of SYO-1 and HT1080 xenografts in athymic mice without obvious detrimental effects. It also mitigated cell proliferation and induced apoptosis in the tumors of mice. This study indicates that increased activity of GSK3β in synovial sarcoma and fibrosarcoma sustains tumor proliferation and invasion through the cyclin D1/CDK4-mediated pathway and enhanced extracellular matrix degradation. Our results provide a biological basis for GSK3β as a new and promising therapeutic target for these STS types.

Keywords
Cell cycle, glycogen synthase kinase 3β, invasion, migration, soft tissue sarcoma

Abbreviations: ABC, avidin-biotin-peroxidase complex; CDK, cyclin-dependent kinase; CRC, colorectal cancer; dUTP, 2′-deoxyuridine-5′-triphosphate; EdU, 5-ethynyl-2′-deoxyuridine; GSK3β, glycogen synthase kinase-3β; PARP-1, poly [ADP-ribose] polymerase 1; PDGF, platelet-derived growth factor; pGSK3βS9, GSK3β phosphorylated in S9 residue; pGSK3βY216, GSK3β phosphorylated in Y216 residue; RTK(s), receptor-type tyrosine kinase(s); S, serine; SR, supplementary reference; STS(s), soft tissue sarcoma(s); WHO, World Health Organization; Y, tyrosine.

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1 | INTRODUCTION

Soft tissue sarcomas (STSs) are a rare tumor type (<1% of all tumors) with an age-adjusted annual incidence of 2.8 per 100,000 people (WHO world standard population) and an age-adjusted incidence rate of 2.4 per 100,000 person-years based on US data. STS arise at nearly all anatomical sites, including the extremities (60% of cases), thorax, abdominal cavity, retroperitoneum, and the head and neck region. More than 50 histological subtypes of STS are defined by the WHO classification, with each having a different treatment response and prognosis. As a result of the wide range of histological subtypes and occurrence at various sites, STS patients are treated by different clinical departments, which ultimately complicates a general overview of STS.

The major histological types of STS in the orthopedic field include synovial sarcoma, fibrosarcoma, rhabdomyosarcoma and undifferentiated sarcoma. Synovial sarcoma is one of the most common STS, accounting for nearly 10% of all primary soft tissue malignancies. It frequently arises in the extremities, particularly the popliteal fossa (knee) in adolescents and young adults. Local recurrence and metastatic disease are common despite initial radical surgery, resulting in a dismal outcome with 5- and 10-year survival rates ranging from 36%-76% and 20%-63%, respectively. Studies have shown only limited survival benefit from adjuvant chemotherapy for high-risk patients with metastatic or residual disease, although synovial sarcoma is relatively chemosensitive compared to other STS. Adult-type fibrosarcoma is a rare and highly aggressive subtype of STS, with most patients aged between 30 and 60 years. Fibrosarcoma is characterized by low sensitivity to radio- and chemotherapy and by a high rate of tumor recurrence. Consequently, the overall prognosis is quite poor, with 10-year survival rates of 60% and 30% for patients with low- and high-grade tumors, respectively.

In the past few years, molecular targeted therapies have been developed for the treatment of many cancer types including STSs. Recently, several targeted agents such as pazopanib (a multi-tyrosine kinase inhibitor) have been developed, together with a new generation of anticancer agents including trabectedin and eribulin for STSs including synovial sarcoma and fibrosarcoma. These agents improved the progression-free and overall survival of STS patients, but showed no improvement over conventional anticancer drugs. Therefore, the discovery of new therapeutic targets is urgently required for the development of efficient, biologically based treatments against STSs.

One emerging target is GSK3β, a serine/threonine protein kinase that maintains homeostasis in normal cells by regulating fundamental biological pathways. Its known functions and involvement in primary pathologies have established GSK3β as a therapeutic target for type 2 diabetes mellitus, neurodegenerative diseases and inflammation. Unlike its effects against several proto-oncogenes (eg, β-catenin, cyclin D1) and mediators of epithelial-mesenchymal transition (eg, snail) in untransformed cells, we previously reported that deregulated GSK3β facilitates the progression of gastrointestinal cancers, glioblastoma and osteosarcoma. This stems from the observation that GSK3β promotes tumor cell survival, proliferation and invasion by modulating distinct tumor suppressor and cell immortality pathways, as well as cell motility. Moreover, GSK3β renders cancer cells insensitive to chemotherapeutics and ionizing radiation. Our observations and other studies establish GSK3β as a common and attractive therapeutic target for a broad spectrum of chronic diseases including cancer. A therapeutic effect from GSK3β inhibition has been shown not only in epithelial but also in hematopoietic, neuronal and musculoskeletal malignancies, including rhabdomyosarcoma and osteosarcoma. Here we explore the expression, activity and putative pathological role of GSK3β in the two major types of orthopedic STSs: synovial sarcoma and fibrosarcoma.

2 | MATERIALS AND METHODS

2.1 | Cell lines and clinical sarcoma samples

This study examined human synovial sarcoma (SYO-1, HS-SY-II, SW982), fibrosarcoma (HT1080) and normal dermal fibroblast (NHDF) cell lines (Table S1). SYO-1 was provided by Dr Ozaki (Okayama University, Japan). HS-SY-II, SW982, HT1080 and NHDF were obtained from RIKEN BRC, ATCC and Takara Bio, respectively, and maintained in DMEM (Wako) supplemented with 10% FBS and Fibroblast Growth Medium (Takara Bio), respectively. Since the precursor cell type for synovial sarcoma is still under investigation and has not been firmly established, we assumed for this work that NHDF were the normal mesenchymal counterpart cells.

We investigated eight patients with synovial sarcoma and four patients with fibrosarcoma who underwent biopsy and/or surgical removal of the primary and/or metastatic tumor in the Department of Orthopaedic Surgery of Kanazawa University Hospital between 2011 and 2018 (Table S2). Tumor specimens were fixed in 10% neutral formalin and embedded in paraffin for histological diagnosis. All patients provided informed consent for the use of their clinical information and tumor specimens for this study. The Kanazawa University Medical Ethics Committee approved all protocols for this study.

2.2 | Western blotting

Cellular protein was extracted using a cell lysis buffer (Cellytic-MT; Sigma-Aldrich). A 30-μg aliquot of protein extract was analyzed by western blotting for the proteins of interest as described previously. Total amount of protein in each sample was monitored by the expression of β-actin. Primary antibodies are listed in Table S3.

2.3 | Immunohistochemical and immunofluorescence staining

Representative paraffin sections of tumors from sarcoma patients (Table S2) were examined for the expression and phosphorylation
of GSK3β and for subcellular localization of β-catenin by the ABC method as per our previous study using antibodies to GSK3β, its fractions phosphorylated in serine (S9) (pGSK3β) and tyrosine (Y)216 residue (pGSK3β-216), and β-catenin (Table S3). Expression and subcellular localization of β-catenin were examined in the sarcoma cell lines by immunofluorescence staining with an antibody shown in Table S3, as described previously.

2.4 | Assays for cell survival, proliferation and apoptosis

Cells were treated with DMSO (Sigma-Aldrich) or with one of the GSK3β-specific inhibitors: AR-A014418 (Calbiochem) or SB-216763 (Sigma-Aldrich) dissolved in DMSO and medium at the indicated concentrations. At the designated time points (0, 24, 48, 72, 96 hours), the relative number of viable cells was measured using a WST-8 assay kit (Wako). Cell proliferation was evaluated following treatment with DMSO or GSK3β inhibitor (25 µmol/L each) for 24 hours by using the Click-iT Plus EdU Alexa Fluor 555 Imaging Kit (Fluoroskan Ascent FL; Thermo Fisher Scientific). Cells positive for EdU in nuclei were observed by fluorescence microscopy (Keyence) and scored following treatment with DMSO, AR-A014418 or SB-216763. Mean percentage of cells positive for nuclear EdU in five microscopic fields was calculated with SD. Cellular apoptosis was measured by western blotting for cleaved caspase-3 and cleaved PARP-1 following treatment of the cells with DMSO or GSK3β inhibitor (25 µmol/L) for 24 hours.

2.5 | RNA interference

To examine the effects of GSK3β expression on cell survival and apoptosis, we used siRNA specific to human GSK3β (5′-GCUCCAGAUCAUGAAAGCUAGAU-3′; GSK-3β Validated Stealth RNAi) and negative control siRNA (Stealth RNAi Negative Control Low GC duplex), both from Invitrogen. We previously confirmed the specificity of GSK3β-specific siRNA. Effect of siRNA on GSK3β expression was monitored by western blotting with an antibody that recognizes both GSK3α and GSK3β (Table S3). The biological effect of an enzyme depends on its biochemical or catalytic activity rather than on its amount. This is also the case with GSK3β since we previously reported that AR-A014418 and SB-216763 inactivate GSK3β in tumor cells within an hour of treatment. In contrast, GSK3β-specific siRNA takes between 48 and 72 hours to substantially, but not completely, deplete GSK3β expression in the same cells. In this study, sarcoma cells were transfected with 20 nanomol/L of either GSK3β-specific or negative control siRNA using Lipofectamine RNAiMAX (Invitrogen). At 96 hours after transfection, the relative number of viable cells was measured as described above.

2.6 | Cell cycle profile analysis

Sarcoma and fibroblast cells were treated with DMSO or 25 µmol/L AR-A014418 for 24 hours. The cells were then trypsinized, washed twice with PBS and fixed with 70% ethanol for 3 hours at −20°C. The fixed cells (1 × 10⁶) were labeled with Muse cell cycle reagent (Millipore) for 30 min at room temperature in the dark and then analyzed for cell cycle fractions by flow cytometry using Muse Cell Analyzer (Millipore). Expression of proteins involved in cell cycle regulation (cyclin D1, CDK4 and CDK6) was examined by western blotting.

2.7 | Collagen gel invasion assay

Cell invasion was investigated using the collagen gel invasion assay. The collagen gel was prepared by Cellmatrix Type I-A (Nitta Gelatin). Sarcoma and fibroblast cells (2 × 10⁵ cells for each cell line) were suspended in 100 µL collagen mixture (3.5 mg/mL) and incubated in 0.5 mL medium containing DMSO or 25 µmol/L AR-A014418 for 2 days. Mean percentage of cells that migrated out of the collagen gel in five microscopic fields was calculated with SD.

2.8 | Gelatin zymography

Sarcoma cells were treated with DMSO or AR-A014418 (25 µmol/L) for 36 hours in serum-free medium. As we described previously, conditioned medium was incubated with SDS sample buffer for 30 min at 37°C. Samples were separated by 10% SDS-PAGE containing 0.005% Alexa Fluor 680-labelled gelatin. Electrophoresed gels were washed in 2.5% Triton X-100 for 2 hours and then incubated in substrate buffer overnight at 37°C. The gel was scanned for secretion and activity of MMP-2 using the LI-COR Odyssey IR imaging system (Lincoln).

2.9 | Animal experiments

Effect of GSK3β inhibition on tumor proliferation was examined for SYO-1 and HT1080 xenograft tumors in BALB/c nude mice (Charles River Laboratories Japan). Cells suspended in Matrigel (BD Bioscience) (1 × 10⁶ cells/100 µL) were injected s.c. Mice assigned to three groups (4 mice each) were given i.p. injections of 75% DMSO or either of the GSK3β inhibitors (2 mg/kg body weight), three times a week for 3 weeks (Figure S1) as we described previously. Assuming that 60% of body weight is accounted by body fluid, a dose of 2 mg/kg body weight corresponds to a concentration of approximately 10 µmol/L in culture media for both inhibitors. Throughout the experiment, all mice were carefully observed for adverse events, while tumors were measured weekly in two
dimensions. Tumor volume (cm$^3$) was calculated using the formula: $0.5 \times a^2 \times b$, where $a$ is the smallest tumor diameter (cm) and $b$ is the largest.

At the point of termination, tumors were removed and tumor weight was measured. Tumors were fixed with 10% neutralized formalin and embedded in paraffin for histopathological, histochemical and immunohistochemical examinations. Paraffin sections of the tumors were stained with hematoxylin and eosin. Sections were immunostained with antibodies against GSK3β, pGSK3β$^{34}$, pGSK3β$^{Y216}$, β-catenin and Ki-67 (Table S3), using the ABC method as we described previously.$^{32,36,38}$ Apoptotic cells in the tumors were evaluated using the In Situ Apoptosis Detection Kit (TUNEL assay kit, M500; Takara Bio) according to the manufacturer’s instructions. Frequency of Ki-67-positive proliferating cells and of TUNEL-positive apoptotic cells in the tumors was calculated as described previously.$^{38}$

All animal experiments were undertaken according to the Japanese national guidelines.$^{39}$ The protocol was approved by the Institute for Experimental Animal Work, Kanazawa University Advanced Science Research Center.

2.10 | Statistical analysis

Data were compared using Student’s $t$ test and ANOVA. $P$ value of <.05 was considered statistically significant.

3 | RESULTS

3.1 | Expression and phosphorylation of GSK3β

Synovial sarcoma, fibrosarcoma and fibroblast cells showed similar basal levels of GSK3β expression. All sarcoma cells showed higher levels of pGSK3β$^{Y216}$ (active form) and lower levels of pGSK3β$^{34}$ (inactive form) compared to NHDF fibroblast cells (Figure 1A). Immunohistochemistry showed expression of GSK3β with Y216 phosphorylation in primary synovial sarcoma and fibrosarcoma, but with less S9 phosphorylation (Figure S2). These findings are consistent with our previous observations in gastrointestinal cancer, glioblastoma and osteosarcoma$^{25,32,36}$ and led us to hypothesize that sarcoma cells may depend on deregulated GSK3β for their survival and proliferation.

One of the most well-recognized consequences of GSK3β inhibition in cells is the stabilization and nuclear translocation of β-catenin, a terminal transducer in the canonical Wnt/β-catenin pathway.$^{58,59}$ We therefore investigated the expression of β-catenin in the sarcoma cell lines and in tumors obtained from patients. Inconsistent with this notion, we found cytoplasmic and nuclear expression of β-catenin (Figures S2 and S3), indicating activation of the β-catenin-mediated pathway in synovial sarcoma cells and clinical tumors. This suggests the absence of intrinsic regulation of β-catenin stability by GSK3β in this sarcoma type. In HT1080 fibrosarcoma cells and patient tumors, most cells showed cytoplasmic expression of β-catenin with scattered cells showing nuclear β-catenin expression.

3.2 | Effects of GSK3β inhibition on sarcoma cell survival and proliferation

To address the above hypothesis of a tumor-promoting role for GSK3β, we examined the effects of GSK3β inhibition on tumor cell survival and proliferation. Viability of all sarcoma cells was reduced by treatment with AR-A014418 or SB-216763 in a dose- and time-dependent manner (Figure 1B). The half-maximal inhibitory concentration (IC$^{50}$) values at 96 hours after administration of AR-A014418 were 16.8, 20.1, 17.9, and 43.7 μmol/L for SYO-1, HS-SY-II, SW982 and HT1080 cells, respectively. The IC$^{50}$ values of SB-216763 for the same cells were 1.36, 20.1, 15.0, and 19.8 μmol/L, respectively. These concentrations are within the range of pharmacological doses for each inhibitor.$^{24,35}$

AR-A014418 and SB-216763 are well established as highly specific against GSK3β$^{24,35}$ and are widely used for studies on the biological functions of this enzyme.$^{58,59}$ We previously demonstrated that both inhibitors attenuate the ability of GSK3β to phosphorylate its substrate in tumor cells.$^{26,58}$ The primary mechanism of action of these inhibitors is to compete with ATP for the ATP-binding domain in GSK3β.$^{24,35}$ Hence they are unlikely to directly influence the phosphorylation of S9 and Y216 residues in GSK3β. However, they may potentially alter the phosphorylation of GSK3β as a consequence of inhibiting enzyme activity. Therefore, we examined changes in the levels of the inactive and active forms of GSK3β in the sarcoma cells following treatment with GSK3β inhibitor. As shown in Figure S4, treatment with AR-A014418 increased S9 phosphorylation but decreased Y216 phosphorylation of GSK3β in the sarcoma cells. β-catenin is a substrate of GSK3β for phosphorylation and subsequent degradation by the ubiquitin-proteasomal system.$^{58,59}$ It was located in the cytoplasm and nucleus of most synovial sarcoma cells, as well as the cytoplasm of fibrosarcoma cells and with scattered nuclear β-catenin-positive cells (Figure S3), despite these cells having active GSK3β (Figure 1A and Figure S4). The same observations were made in clinical tumors, with active GSK3β observed in synovial sarcoma and fibrosarcoma samples obtained from patients (Figure S2). This paradoxical subcellular localization of β-catenin and the level of its expression were unchanged by treatment with the GSK3β inhibitor (Figures S3 and S4).

As early as 24 hours after administration, the GSK3β inhibitors significantly decreased the number of EdU-positive proliferating cells (Figure 2A,B and Figure S5) and increased the fraction of cleaved PARP-1 and caspase-3 (Figure 2C) in the sarcoma cells but not in the fibroblasts. Similar effects were observed in the sarcoma cells following GSK3β RNA interference (Figure 3 and Figure S6). The biological effect of RNA interference depends on the efficiency of siRNA transfection and on the subsequent knockdown of the target molecule. Therefore, we believe the pharmacological inhibitors more quickly and efficiently inhibit GSK3β activity in cells, leading
to a stronger biological effect on cells compared to treatment with GSK3β-specific siRNA. These results indicate that both activity and expression of GSK3β are required for the survival and proliferation of synovial sarcoma and fibrosarcoma cells, thereby suggesting a potential tumor-promoting role for this kinase.

3.3 | Effects of GSK3β inhibition on the cell cycle profile and on its regulation

Flow cytometry analysis revealed an increased G0/G1 fraction in all sarcoma cells treated with 25 µmol/L AR-A014418 for 24 hours, indicating the induction of G0/G1-phase cell cycle arrest (Figure 4A,B). Many chemotherapeutic drugs cause cancer cells to undergo growth arrest in the G1 cell cycle phase.40 In mammalian cells, cell cycle transition from G1 to S-phase is mediated by CDK/cyclin complexes.41,42 Treatment with 25 µmol/L AR-A014418 for 24 hours consistently decreased the expression of cyclin D1, CDK4 and CDK6 in the sarcoma cells (Figure 4C).

3.4 | GSK3β inhibitor suppresses the invasion of sarcoma cells via MMP-2 inactivation

Prevention of invasion and metastasis by sarcomas is indispensable for the successful treatment and care of patients. Curative treatment for synovial sarcoma patients with distant metastasis is rarely achievable, with the exception of late and resectable oligometastatic disease. Such patients undergo chemotherapy with palliative intent.11 The most important way to improve prognosis in
To examine the possible contribution of GSK3β to invasive tumor growth, the invasive capacity of sarcoma and fibroblast cells was monitored and scored using the collagen gel invasion assay. All sarcoma cells invaded out of the collagen gel, whereas the fibroblast cells scarcely invaded. Treatment with 25 µmol/L AR-A014418 for 48 hours significantly attenuated the invasive capacity of sarcoma cells (Figure 5A,B). It was reported that various sarcomas, including synovial sarcoma and fibrosarcoma, express MMP-2.43,44 This enzyme plays a crucial role in tumor invasion and metastasis through degradation of extracellular matrix. In the present study by gelatin zymography, compared to NHDF fibroblasts, most sarcoma cells...
expressed intermediate and active forms of MMP-2. Treatment of the sarcoma cells with 25 µmol/L AR-A014418 for 36 hours decreased the expression of these forms, as well as pro-MMP-2 secretion (Figure 5C).

3.5 | Effect of GSK3β inhibitors on xenograft tumors in mice

The efficacy of GSK3β inhibitors against tumors and their safety in rodents are prerequisites for clinical translation of GSK3β-targeted therapy. 25-27 Although GSK3β inhibition induced apoptosis more efficiently in HS-SY-II and SW982 than SYO-1 cells (Figures 2C and 3D), it attenuated the survival and proliferation of SYO-1 cells as efficiently as for the other two cell lines (Figures 1B, 2B, and 3C). In several recent studies, SYO-1 was well characterized for the generation of xenograft tumors. 25-27 HT1080 was the only fibrosarcoma cell line investigated in this study, hence we used both SYO-1 and HT1080 cells for the xenograft experiments. Administration of GSK3β inhibitors in mice significantly reduced xenograft tumor volume as early as 2 weeks after treatment initiation for HT1080 tumors and 3 weeks for SYO-1 tumors (Figure 6A). At the time of treatment termination (4 weeks), tumors from GSK3β inhibitor-treated mice weighed significantly less than those from control mice (Figure 6B). No obvious side effects or adverse events were observed in the mice following the respective treatments.

We compared the levels of active and inactive forms of GSK3β in the xenograft tumors between mice treated with DMSO or with either of the GSK3β inhibitors. Similar to the sarcoma cells in culture (Figure S4), immunohistochemistry showed that treatment of xenograft tumors with GSK3β inhibitors enhanced S9 phosphorylation and reduced Y216 phosphorylation, but did not affect GSK3β expression (Figure S7). These results indicate that GSK3β activity was inhibited in the tumor cells. As observed in the cultured cells (Figure S3), no difference was found in the expression level and subcellular localization of β-catenin between tumors from control (DMSO) or GSK3β inhibitor-treated mice (Figure S7). Immunohistochemical and histochemical examinations revealed that the number of Ki-67-positive proliferating cells and of TUNEL-positive apoptotic cells in tumors from GSK3β inhibitor-treated mice was significantly lower and higher, respectively, than in the control group treated with DMSO (Figure 6C,D and Figure S8).
Several potential molecular therapeutic targets have emerged recently for STSs. Unfortunately, the clinical trials that target them have shown little benefit for patients with refractory STSs, including synovial sarcoma and fibrosarcoma. This has led us to investigate whether GSK3β could be a useful target for the treatment of these sarcoma types. The present study indicates that synovial sarcoma and fibrosarcoma cells preferentially express the active form of GSK3β (pGSK3βY216). Our group and others have reported that pGSK3βY216 promotes tumor cell survival, proliferation and invasion in several cancer types. In line with this observation, we show here for the first time that inhibiting the activity or expression of GSK3β can reduce cell survival, proliferation and invasion of synovial sarcoma and fibrosarcoma cells, as well as inducing apoptosis. Furthermore, we confirmed the efficacy of GSK3β inhibitors against synovial sarcoma and fibrosarcoma xenograft tumors in mice. Together with previous studies, these results suggest that sarcoma cells depend on active GSK3β for their survival, proliferation and invasion, thereby reinforcing the potential importance of GSK3β as a common target for the treatment of different sarcoma types including synovial sarcoma and fibrosarcoma, despite having heterogeneous histology and biology.

An important observation regarding this potentially new therapeutic approach is the negligible effect of GSK3β inhibition on the
growth or apoptosis of untransformed, normal fibroblast (NDHF) cells. This is consistent with previous studies showing that GSK3β inhibition did not influence the survival or growth of other non-neoplastic cells including human embryonic kidney (HEK293) cells, embryonic lung fibroblasts (WI38), mammary epithelial cells (HMEC) and mouse embryonic fibroblasts (NIH-3T3). Compared to sarcoma cells, NHDF cells were found to show a lower level of the active pGSK3βY216. No harmful effects of the GSK3β inhibitors were apparent in NHDF cells, nor in the mice with tumor xenografts in this study. This finding together with previous studies on the different subtypes (alveolar and embryonal) of rhabdomyosarcoma further supports the potential use of GSK3β inhibitors for the treatment of STSs in the clinical setting.

The highly proliferative and invasive nature of STS cells promotes their progression and results in incomplete surgical removal of the tumor and failure of chemotherapy. In the present study, inhibition of GSK3β induced GO/G1-phase cell cycle arrest and decreased the expression of cyclin D1, CDK4 and CDK6. This is responsible for the anti-proliferative effect of GSK3β inhibitors on sarcoma cells, as we observed previously in gastrointestinal and pancreatic cancers. It was reported that CDK4 and CDK6 are activated in a complex with cyclin D1 in various sarcoma types, including STSs, thus facilitating their progression. Therefore, our results suggest that synovial sarcoma and fibrosarcoma cells depend on deregulated GSK3β for their survival and proliferation via cyclin D1 and CDK4/6-mediated cell cycle progression and evasion of apoptosis. We also showed that GSK3β inhibition attenuated the invasion of collagen gel by sarcoma cells. This was associated with a decrease in the secretion and activity of MMP-2. Previous studies reported that aberrant expression and activity of MMP-2 in various sarcomas including synovial sarcoma and fibrosarcoma imparts invasive and metastatic properties to the tumor cells. We previously showed in pancreatic cancer and glioblastoma cells that GSK3β increases the expression and activity of MMP-2 via induction of the pathway mediated by focal adhesion kinase, Rac1 and c-Jun N-terminal kinase, thus conferring invasive ability. The present results and the previous studies support the contention that G0/G1-phase cell cycle arrest and suppression of tumor cell invasion underpin the therapeutic effects of GSK3β inhibition in synovial sarcoma and fibrosarcoma.

One of the most recognized consequence of GSK3β inhibition in normal cells is stabilization and nuclear translocation of β-catenin, thus provoking Wnt/β-catenin signaling. As we discussed previously, the Wnt/β-catenin pathway participates in osteogenesis and in bone homeostasis by promoting the osteoblastic cell lineage and countering the osteoclastic cell lineage. Based on this mechanism, a previous study attempted to treat osteoporosis using GSK3β inhibitors. In contrast to its role in normal osteogenesis, several studies including ours reported that the β-catenin-mediated pathway attenuates the development and progression of osteosarcoma, undifferentiated sarcoma and rhabdomyosarcoma. In contrast to the reported therapeutic efficacy of GSK3β inhibition against various cancer types including these sarcomas, it has been reported that GSK3β inhibition maintains...
the stemness capacity of embryonic, hematopoietic and mesenchymal stem cells, the latter consistent with a previous study showing the development of undifferentiated sarcoma via inactivation of the Wnt pathway in mesenchymal stem cells. Taken together, these findings suggest the Wnt/β-catenin pathway functions as a tumor suppressor in osteosarcomas, rhabdomyosarcomas and probably undifferentiated sarcomas, all of which are encountered in the orthopaedic field.

In contrast to the above sarcoma types, the present study of synovial sarcoma cells and clinical tumors found nuclear accumulation of β-catenin. Several previous studies reported activation of Wnt/β-catenin signaling in synovial sarcoma and considered this to be a potential therapeutic target. However, none of these studies showed that inhibition of GSK3β activates β-catenin signaling in synovial sarcoma. Therefore, unlike our previous study on osteosarcoma, the molecular mechanism underpinning the therapeutic effect of GSK3β inhibition against synovial sarcoma does not appear to involve an effect on the β-catenin-mediated pathway.

GSK3β activity in untransformed cells is negatively regulated by phosphorylation of its S9 residue via the Akt- and Wnt-mediated pathways. It is therefore important to address whether these proto-oncogenic pathways are inactive in synovial sarcoma and accumulation of β-catenin in the sarcoma cells and their xenograft tumors. Several previous studies reported activation of Wnt/β-catenin signaling in synovial sarcoma and considered this to be a potential therapeutic target. However, none of these studies showed that inhibition of GSK3β activates β-catenin signaling in synovial sarcoma. Therefore, unlike our previous study on osteosarcoma, the molecular mechanism underpinning the therapeutic effect of GSK3β inhibition against synovial sarcoma does not appear to involve an effect on the β-catenin-mediated pathway.

**FIGURE 6** Effect of glycogen synthase kinase-3β (GSK3β) inhibitors on the size and weight of synovial sarcoma SYO-1 and fibrosarcoma HT1080 xenograft tumors in mice. A, Tumor size was measured weekly and the volume calculated. B, Mean weight of the tumors removed at necropsy. Open, gray and closed columns indicate treatment with DMSO, 2 mg/kg AR-A014418 or SB-216763, respectively. (A, B) Asterisks denote a statistically significant difference in tumor volume and weight compared to mice treated with DMSO (*P < .05; **P < .01). C and D, Scores for Ki-67-positive proliferating cells (C) and TUNEL-positive apoptotic cells (D) in tumors removed from mice following treatment for 3 wks with either DMSO, AR-A014418 or SB-216763. As shown in Figure S8, in each tumor, the total number of nuclei in a high-power microscopic field was counted and scored for Ki-67-positive and TUNEL-positive cells, respectively. Mean scores of Ki-67-positive and TUNEL-positive cells in five microscopic fields were then calculated with SD and statistically compared between the tumors of mice treated with DMSO (open column), AR-A014418 (gray column) or SB-216763 (closed column). Asterisks denote a statistically significant difference in Ki-67-positive and TUNEL-positive cells compared to mice treated with DMSO (*P < .005).
fibrosarcoma in which GSK3β was found in this study to be active. We previously compared the expression and phosphorylation-dependent activity of GSK3β, Akt and β-catenin in colorectal cancer (CRC), where the Akt and Wnt pathways are frequently activated. Similar to the present study of synovial sarcoma and fibrosarcoma, we found higher GSK3β expression and Y216 phosphorylation and lower S9 phosphorylation in CRC cell lines and primary tumors compared to non-neoplastic HEK293 cells and normal colorectal mucosa tissue, respectively. However, no causative association was found between GSK3β activity (Y216 phosphorylation) and Akt activity (indicated by threonine 307 and S473 phosphorylation), or between GSK3β activity and nuclear accumulation of β-catenin in CRC cells and primary tumors. This suggests that GSK3β activity in CRC is unrelated or irrelevant to the Akt and Wnt pathways. Several studies have shown activation of the Wnt and Akt-mediated pathways in synovial sarcoma, but little is known about the involvement of these pathways in fibrosarcoma. Analysis of the relative impacts of the GSK3β-, Akt- and Wnt-mediated pathways in the same tumor type is essential to gain a deeper understanding of the disease mechanism and thus to develop more efficient therapeutic strategies. However, it was beyond the scope of the present study to determine which of these pathways predominates in synovial sarcoma.

Receptor-type tyrosine kinase inhibitors are the mainstay of preclinical studies and clinical trials of targeted therapy against cancer, including sarcomas. Recent preclinical studies of various STS types including synovial sarcoma have evaluated the therapeutic efficacy of TAS-115, which targets c-Met and PDGF receptor, and of nintedanib which is a potent inhibitor of the PDGF, vascular endothelial growth factor and fibroblast growth factor receptors. These RTK inhibitors were associated with a decrease in Akt activity in the tumor cells. The latter study also showed more efficient anti-tumor effects of nintedanib against synovial sarcoma SYO-1 and HS-SY-II cells and their xenograft tumors compared to the other RTK inhibitors imatinib (inhibitor of c-Kit, PDGF receptor and ABL tyrosine kinases) and MGCD516 (sitrativatinib targeting c-Kit, PDGF receptors, c-Met and Axl) in clinical and preclinical studies of synovial sarcoma. Other studies have tested the preclinical efficacy of multitarget RTK inhibitors (ABT-869 and ABT-348) against fibrosarcoma HT1080 cells and their xenograft tumors. The efficacy of Akt, RTK and GSK3β inhibitors against STSs has yet to be determined and is an important step in the future development of efficient targeted therapies against this tumor type.

In conclusion, this study demonstrates that deregulated activity of GSK3β in synovial sarcoma and fibrosarcoma is responsible for tumor cell proliferation via cyclin D1/CDK4-mediated cell cycle progression, and for tumor cell invasion via enhanced extracellular matrix degradation. The present results therefore suggest that targeting of GSK3β in tumor cells is a promising therapeutic strategy with widespread application for synovial sarcoma, fibrosarcoma, osteosarcoma and rhabdomyosarcoma. These sarcoma types are all devastating diseases with different histology, biology, clinical manifestation and response to therapies. Prior to the clinical use of GSK3β-targeted therapy, critical prerequisites are firstly to test the effect of GSK3β inhibitors against patient-derived primary sarcoma cultures and xenograft tumors (PDXs), and secondly to clarify the underlying molecular mechanism of action.

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DISCLOSURE
Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.