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Original Articles

Hsp90 inhibition by AUY922 as an effective treatment strategy against myxoid liposarcoma

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ABSTRACT

Liposarcoma is one of the most common soft tissue sarcomas in adults. Recognized histological subtypes include well differentiated/dedifferentiated liposarcoma (WD/DDLS), myxoid liposarcoma (MLS) and pleomorphic liposarcoma. Currently, there are no proper subtype-specific treatments due to the genetic, histological and clinical heterogeneity of the liposarcoma subentities. In the past decade, the rising understanding of the various genetic and molecular aberrations in liposarcoma led to the development of novel alternative therapeutic strategies. One such therapy is the inhibition of the heat shock protein 90 (Hsp90) which is overexpressed in liposarcomas. In this study, we dissect the functional role of a novel potent Hsp90 inhibitor NVP-AUY922 (AUY922) in different cell lines of myxoid (MLS402, MLS1765) and undifferentiated (SW872) liposarcomas. We show that compared with 17-AAG treatment, lower concentrations of AUY922 achieve markedly cytotoxic effects on tumor cell viability. Combination treatment of AUY922 (20 nM) with Doxorubicin (300 nM) yielded a further reduction in cell viability in comparison to Doxorubicin alone. *In vivo*, we document an inhibition of tumor growth after AUY922 treatment. Further analyses revealed that Hsp90-inhibition induces apoptotic cell death and cell cycle arrest. In addition, we report striking perturbations of subtype-specific pattern in Raf/MEK/ERK and PI3K signaling after AUY922 application. In conclusion, our results provide evidence that Hsp90-inhibition by AUY922 may be a promising alternative therapeutic strategy for myxoid liposarcoma patients.

Introduction

An estimated incidence of 11,930 new cases of soft tissue sarcomas (STS) has been reported in the United States in 2015 [1]. Approximately 20% of all STS are liposarcomas, representing the most common group of malignant mesenchymal neoplasms in adults [2]. Based on specific morphology and cytogenetic aberrations, three subtypes of liposarcoma are recognized by the current World Health Organization (WHO) classification of soft tissue and bone tumors. In decreasing order of frequency, atypical lipomatous tumor/well differentiated liposarcoma (ALT/WDLS)/dedifferentiated liposarcoma (DDLS), myxoid liposarcoma (MLS) and pleomorphic liposarcoma (PLS) are the main subtypes [3].

Despite their rare occurrence, liposarcomas have a considerable morbidity and mortality impact with particularly poor prognosis based on their histological subtype, the anatomical location and tumor burden [4,5]. The extent of cell differentiation, ranging from low-grade tumors with high local recurrence rate but no metastatic potential (WD/DDLS) to highly metastasizing tumors (the round cell variant of MLS or PLS), remains the most notably determinant of clinical course and ultimate prognosis. WDLS and PLS are largely chemo-resistant, while MLS is known to be sensitive to radiation and cytotoxic chemotherapy. Both histological and genetic heterogeneity have challenging implications for the treatment of aggressive local or metastatic disease.

Surgery, radiotherapy and conventional cytotoxic chemotherapy, the mainstay of systemic first-line therapy of liposarcoma, may prevent the recurrence and delay temporarily the disease progression, but they have no considerable impact on long-term survival. The poor five year survival of approximately 50% [2,6] emphasizes the need of more effective adjuvant or neoadjuvant therapies.

Current typical first-line chemotherapy agents for unresectable and metastatic liposarcoma consist of single agent anthracyclines (Doxorubicin) or anthracycline-based combination products

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(Doxorubicin with Ifosfamide or Dacarbazine). For second-line treatment non-anthracycline combinations (Gemcitabine and Docetaxel) are used. The aim of novel therapies is to target specific aberrant genetic or molecular pathways which inevitably require the understanding of the disease biology. Recent clinical trials provide a promising therapeutic potential of novel substances in the neoadjuvant setting for liposarcoma such as Trabectedin (MLS) and Flavopiridol (WD/DDLS).

A novel promising therapeutic strategy for liposarcoma is the targeting and inhibition of the heat shock protein 90 (Hsp90) [7,8]. Our preliminary work revealed the expression of Hsp90 in liposarcoma subtypes. Hsp90 is a highly conserved ATP-dependent multichaperone protein which is required for the stability, post-translational modification and function of multiple client proteins. The major client proteins of Hsp90 are implicated in cell growth and differentiation (EGFR, IGFR, HER-2/neu, AKT, Raf, BCR-ABL), anti-apoptotic effects (AKT, p53) and progression of the cell cycle (CDK4, cyclin D1). In addition, Hsp90 plays an important role in tissue invasion, metastasis (Met, FAK) and angiogenesis (VEGF). Considering that Hsp90 is involved in multiple signaling pathways including Raf/MEK/ERK and PI3K/AKT, its inhibition is expected to result in deficient modulation of several components of these pathways for ultimately achieving anti-cancer effects in liposarcoma patients.

A variety of selective Hsp90 inhibitors have been developed. Recently, a small molecule NVP-AUY922 (AUY922), a resorcinylic isoxazole amide, has shown promising anti-tumor activity in preclinical and phase I clinical trials [9]. AUY922 is a purine-scaffold derivative which mimics the ATP/ADP binding interface at the N-terminus of Hsp90 inhibiting potently the naive activity of the multichaperone and complexed client proteins [10].

This paper aims to evaluate if Hsp90-inhibition could be considered as a novel potential therapeutic strategy in liposarcoma. We used several approaches including cell viability studies, an apoptosis assay and immunoblotting to investigate the dysregulation of components of the Raf/MEK/ERK and PI3K/AKT signaling pathways in response to the Hsp90 inhibitor AUY922. Furthermore, we determined the effect of Hsp90 inhibition on tumor growth *in vivo*.

Materials and methods

Chemical compounds

The Hsp90 inhibitor NVP-AUY922 (AUY922) (5-(2,4-Dihydroxy-5-isopropyl-phenyl)-N-ethyl-4-[4-(morpholinomethyl)-phenyl]isoxazole-3-carboxamide) was kindly provided by Novartis (Basel, Switzerland). 17-AAG (17-(Allylamino)-17-demethoxygeldanamycin) and Doxorubicin were purchased from Sigma-Aldrich (Taufkirchen, Germany) or Selleckchem (Munich, Germany), respectively. For *in vitro* studies, AUY922, 17-AAG and Doxorubicin were dissolved in 100% dimethyl sulfoxide (DMSO) to 250 mmol/l, 0.85 mmol/l or 1 mmol/l stock solutions, respectively, and stored at −20 °C. The stock solution was dissolved to 250 µmol/l for AUY922 and to 100 µmol/l for 17-AAG and Doxorubicin with cell culture medium RPMI 1640 or DMEM.

Tumor cell lines and cell culture

The human MLS cell lines MLS1765-92 (here: MLS1765) and MLS402-91 (here: MLS402) are a generous gift of Prof. Dr. Pierre Aman (University of Gothenburg, Sweden). The human parental fibrosarcoma cell line HT1080 was kindly provided by Prof. Dr. Helge Taubert (University of Erlangen-Nuremberg, Germany). The only available cell line of a distinct liposarcoma subentity – however, without MDM2 amplification – used was the human undifferentiated liposarcoma cell line SW872. The latter and the human colon cancer cell line HCT116 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The identity of SW872, HT1080 and HCT116 cells was confirmed by Multiplex human Cell Line Authentication Test (MCA, Multiplexion, Heidelberg, Germany). All cell lines were stored in liquid nitrogen and cultured at 37 °C in humidified atmosphere and 5% CO_2 in RPMI 1640 medium (Gibco®, Life Technologies™, Carlsbad, USA) supplemented with 10% fetal calf serum, 1% penicillin (100 U/ml) and streptomycin (100 µg/ml) (PAN Biotech, Aidenbach, Germany). HT1080 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing same supplements. All cell lines were free of mycoplasma (PCR based system).

Genotyping of myxoid liposarcoma cell lines

Myxoid liposarcoma harbors a specific t(12;16)(q13;p11) translocation resulting in a FUS/DDIT3 fusion gene. Therefore, both cell lines MLS402 and MLS1765 were characterized for a FUS/DDIT3 gene isoform using real-time PCR. MLS402 and MLS1765 cells each showed two different banding patterns. Type I (MLS402) and type VIII (MLS1765) FUS/DDIT3 isoforms were confirmed by Prof. Dr. Pierre Aman (University of Gothenburg, Sweden) (Fig. S1). The exact karyotypes of MLS402 and MLS1765 have been described earlier [11]. The mesenchymal origin of MLS402 and MLS1765 cells was validated by Western blotting (Fig. S1).

Crystal violet assay

To determine the cytotoxic effect of the Hsp90 inhibitors on cell growth *in vitro*, we used the crystal violet cell viability assay. Cells (7.5×10^3/well) were seeded in 200 µl of growth medium in 96-well plates. After an overnight attachment period, cells were exposed to varying concentrations (0.1, 10, 20, 40, 60, 100 and 1000 nM) of AUY922 or 17-AAG for 6, 24 and 48 hours. For treatment combination experiments, we followed three protocol variants: (i) cells were simultaneously treated, (ii) pretreated with AUY922 for 24 hours followed by Doxorubicin exposure after discarding AUY922 or (iii) pretreated with AUY922 for 24 hours and subsequently treated with Doxorubicin for further 24 or 48 hours (AUY922: 20 nM; Doxorubicin: 100 nM or 300 nM). After washing with PBS, the cells were incubated on a shaker for 15 minutes with 50 µl crystal violet staining solution (20% methanol) at room temperature (RT). Washed and dried cells were resorbed in 100% methanol and shaken for 15 minutes. The optical density was measured at 620 nm using a multilabel reader (Perkin Elmer, Rodau, Germany). All studies were performed in quadruplicate or quintuplicate. The average absorbance of untreated cells was defined as 100% cell viability.

Annexin V/PI staining

Apoptosis was detected using the Annexin-V-FLUOS staining Kit (Roche Diagnostics, USA). Cells were stimulated with 100 nM AUY922 for 24 or 48 hours. Floating and adherent cells were harvested by trypsinization and washed twice with PBS before staining with 100 µl annexin V/PI solution, consisting of 20 µl Annexin-V-FLUOS labeling reagent (20 µg/ml), 20 µl propidium iodide solution (50 µg/ml) and 1 ml HEPES buffer, for 15 minutes at RT in the dark. Cell suspension was diluted by adding appropriate amount of incubation buffer and analyzed in a flow cytometer BD FACSCanto® II (BD Biosciences, Germany) and FlowJo software. Annexin-V binding by phosphatidylserine (PS)-exposing cells was defined as apoptosis. Unstained cells served as control.

RNA extraction, cDNA synthesis and real-time PCR analysis

Total cellular RNA was extracted from MLS402, MLS1765, SW872 and HT1080 cells treated with 100 nM AUY922 for 6, 24 or 48 hours using RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized using QuantiTect® Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Absolute quantitative real-time PCR for fus-ddit3 and p21/waf1 was performed in a final volume of 25 µl using the CFX 96 TouchTM Real-Time PCR Detection system (Bio-Rad) and CFX manager software (Bio-Rad) for threshold cycle number determination using the comparative ΔCt method [12]. Primer sequences for fus-ddit3 were fus_for 5′-CAGAGCTCCCAATCGTCTTACGG-3′ and ddit3_rev 5′-GAGAAAGGCAATGACTCAGCT GCC-3′ and for p21/waf1 sense 5′-GGCAGACCAGCATGACAGATT-3′ and anti-sense 5′-GCGGCCAGGGTATGTA CATGA-3′. Each reaction consisted of 1 µl of cDNA, 12.5 µl of SYBR green mix (QuantiFast SYBR Green PCR Kit, Qiagen), 2.5 µl of each primer (10 pmol/µl) and 6.5 µl of RNAse free water. The thermal cycling conditions were: 95 °C for 1 min, followed by 45 cycles at 95 °C for 10 s, 65 °C (fus-ddit3) or 62 °C (p21/waf1) for 10 s and 72 °C for 30 s. β2-microglobulin (β2m) (sense: 5′-TGACTTTGTCACAGCCCAAGATA-3′; antisense: 5′-AATC CAAATGCGGCATCTTC-3′) expression served as an endogenous control to normalize the data. All samples were analyzed in triplicate with the threshold cycle number as average.

Western blotting analysis

Cells were lysed in urea lysis buffer (4 M urea, 0.5% SDS, 65.5 mM Tris-Base) supplemented with 1 nM Phenylmethylsulfonylfluorid (PMSF) and 1:100 Protease Inhibitor Cocktail Set III (Calbiochem). The protein concentration of cell lysates was determined by using Bio-Rad DC protein Assay (BioRad Laboratories, CA). Samples containing equal amounts of protein (30 µg) were then separated by 10% or 12% denaturing SDS-PAGE and transferred onto nitrocellulose membranes. Blots were probed overnight at 4 °C with specific primary antibodies anti-AKT (1:1000), -biotin (1:5,000), -caspase 3 (1:1000), -E-cadherin (1:1000), MEK1/2 (1:1000), -p44/42 MAPK (1:1000), -PARP (1:1000), -p21 Waf1/Cip1 (1:2000), -pAKT^{Ser473} (1:2000), -p-Histone3^{Ser10} (1:1000), -p-MEK$^{12Ser217/221}$ (1:1000), -p-p44/42 MAPK$^{T202/Y204}$ (1:1000), -vimentin (1:1000) (Cell Signaling Technology Inc., USA), -Chk1(1:500), -cyclin B1 (1:1000), -cytokeratin 8 (1:1000), -Hsp90 (1:2000), -Hsp70 (1:1000) (Santa Cruz Biotechnology Inc., USA), -GAPDH-HRP (1:20,000; Abnova, USA) and -Histone3 (1:2000; Active motif, Belgium) and secondary horseradish peroxidase-coupled antibodies anti-mouse or anti-rabbit (1:20,000; Pierce, Rockford, IL). Bound antibodies were visualized

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