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HSP72 functionally inhibits the anti-neoplastic effects of HDAC inhibitors

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ABSTRACT

Background: The anticancer effects of histone deacetylase inhibitors (HDACi) vary between patients, and their molecular mechanisms remain poorly understood. Previously, we have identified heat shock 70 kDa protein 1A (HSPA1A, also known as HSP72) as the most overexpressed protein in valproic acid (VPA)-resistant cell lines. KNK437, an inhibitor of heat shock proteins, enhanced the cytotoxic effects of not only VPA but also vorinostat, another HDACi. However, the mechanisms underlying the role of HSP72 in resistance against HDACi remain largely unknown.
Objective: The purpose of this study was to identify the mechanisms underlying the role of HSP72 in HDACi resistance.
Methods: We established an HSP72-overexpressing Jurkat cell line and used it to assess the functional role of HSP72 following treatment with the HDACi vorinostat and VPA.
Results: HDACi-induced apoptosis, assessed using annexin V assays, sub-G1 fraction analysis, and PARP cleavage, was significantly lower in HSP72-overexpressing cells than in control cells. The HDACi-induced upregulation in caspase-3, −8, and −9 activity, as well as the HDACi-induced reduction in mitochondrial membrane potential, were also suppressed following HSP72 overexpression. The basal expression levels of Bcl-2, phosphorylated Bad, and XIAP increased in HSP72-overexpressing cells, whereas HDACi-induced Bid truncation and the suppression of Bad expression. Furthermore, vorinostat-induced histone hyperacetylation was also diminished in HSP72-overexpressing cells.
Conclusion: These findings clearly demonstrate that HSP72 inhibits HDACi-induced apoptosis.

1. Introduction

Histone deacetylase inhibitors (HDACi) enhance the acetylation of both histones and non-histone proteins, and induce apoptosis. They are potential therapeutic agents for the treatment of lymphoid neoplasms, but, with an overall remission rate of approximately 30%, they exhibit limited effectiveness [1–4]. The molecular mechanisms underlying the variability in HDACi sensitivity remain largely unknown; however, HR23B, a candidate biomarker for HDACi sensitivity [5], has been shown to regulate HDACi-induced apoptosis and autophagy [6].

Previously, while attempting to clarify mechanisms of resistance to HDACi, we analyzed the proteomic features of 33 lymphoid cell lines. We reported that a higher baseline expression level of heat shock 70 kDa protein 1A (HSPA1A; also known as HSP72) was associated with HDACi resistance, and that the HSP inhibitor KNK437 enhanced HDACi-induced apoptosis [7].

Under normal physiological conditions, HSP72 expression is minimal or absent. However, the expression of this HSP70 family member can be induced by different stresses, and is constitutively elevated in human tumors. HSP72 inhibits several components of the apoptotic pathway, including the proapoptotic Bcl-2 family proteins, thus conferring a survival advantage. Furthermore, in some cell types, the HSP72 expression level correlates with poor prognosis and chemoresistance to some therapeutic agents [8]. However, HSP72 expression in renal cancer cells, which is significantly higher than that in normal renal cells, is reportedly a favorable prognostic factor in patients with renal cancer [9], and HSP72 expression in osteosarcoma is predictive of a favorable response to neoadjuvant chemotherapy [10].

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Treatment with HDACis has been shown to induce HSP72 expression: LAQ824 upregulates HSP72 expression in peripheral blood mononuclear cells (PBMCs) from cancer patients and in cutaneous T-cell lymphoma (CTCL) tumor tissues [11], JNJ-26481585 induces HSP72 expression in both primary myeloma cells and established myeloma cell lines [12], and suberoylanilide hydroxamic acid (SAHA, also known as vorinostat) reportedly increases HSP70 expression in the cerebral cortex [13]. Additionally, HDACis were shown to promote both Sp1 acetylation and the association of Sp1 with the histone acetyltransferase p300, as well as the binding of p300 to the Hsp70 promoter [14]. Furthermore, Kaiser et al. [15] showed that treating acute myeloid leukemia and acute lymphoblastic leukemia cell lines with a combination of the HSP72 inhibitor pifithrin-μ and vorinostat resulted in significantly lower cell viability than treatment with either drug alone, although the mechanisms underlying this effect remain unclear.

In this study, we examined the role of HSP72 expression in HDACi resistance by establishing an HSP72-overexpressing Jurkat cell line, treating these cells with HDACi, and assessing the functional role of HSP72.

2. Materials and methods

2.1. Establishment and characterization of an HSP72-overexpressing cell line

Jurkat cells, which express low levels of HSP72 and are sensitive to SAHA and valproic acid (VPA) [7], were cultured at 37 °C in 5% CO_2. An HSP72 cDNA clone was purchased from DNAFORM (Yokohama, Japan) and subcloned into a pcDNA3.1 expression vector. Jurkat cells were then transfected with either pcDNA3.1-HSP72 or pcDNA3.1 empty vector using Lipofectamine LTX with PLUS reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Stably transfected cells were selected by adding 1000 μg/ml G418 to the growth medium (WAKO, Tokyo, Japan).

HSP72 mRNA expression was confirmed using real-time RT-PCR and the ABI 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA). Briefly, total RNA was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany), and then cDNA synthesis was performed using the SuperScript First-Strand Synthesis system (Invitrogen), according to the respective manufacturers' protocols. Next, qRT-PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) and an ABI 7300 instrument. RT-PCR reactions contained synthesized cDNA and TaqMan Gene Expression Assay components (Hs00359147_s1 for HSPA1A, Hs99999905_m1 for GAPDH; Applied Biosystems). The HSP72 expression in each sample was normalized to that of *GAPDH*.

HSP72 protein expression was examined by flow cytometry and by western blotting, using a FITC-conjugated anti-HSP72 antibody (clone C92F3A-5, Enzo Life Sciences, Farmingdale, NY). For flow cytometry, cells were fixed with methanol and permeabilized with phosphate-buffered saline (PBS)-T (PBS containing 0.1% Triton X-100). Cells were then washed with PBS, stained with either anti-HSP72-FITC or isotype control antibody, and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The relative mean fluorescence intensity (rMFI) of HSP72 was calculated by dividing the mean fluorescence intensity (MFI) of

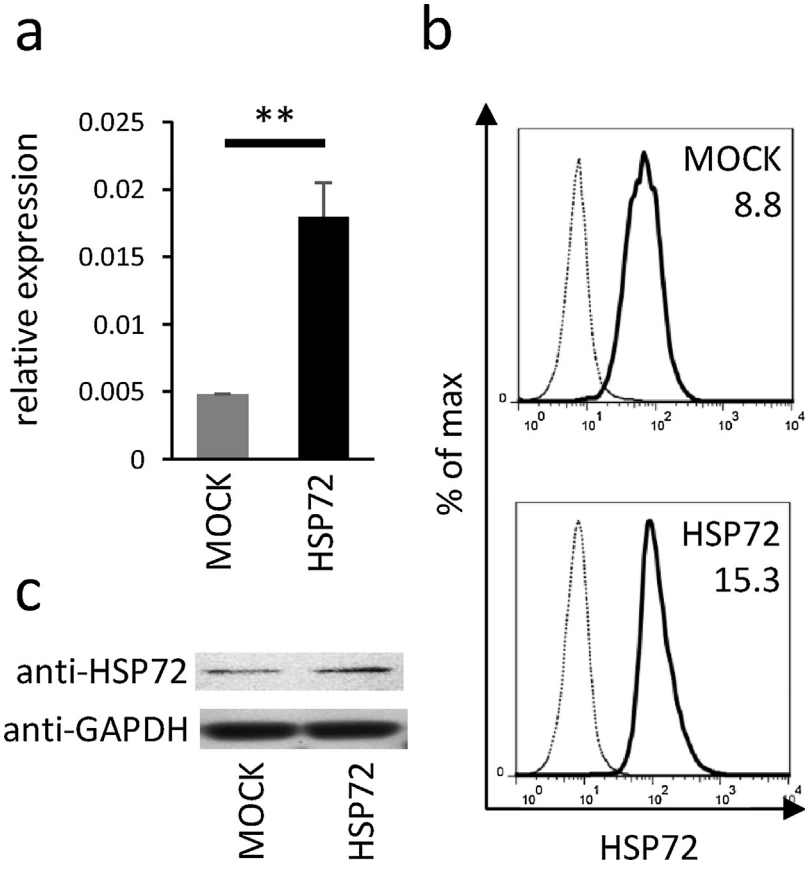

Fig. 1. Characterization of HSP72-overexpressing cells. (a) HSP72 mRNA expression for MOCK and HSP72-transfected Jurkat cells, normalized to *GAPDH* expression. Gray bar: Jurkat-MOCK cells; black bar: Jurkat-HSP72 cells. (b–c) HSP72 protein expression. (b) Flow cytometry analysis of HSP72 expression in Jurkat-MOCK (upper panel) and Jurkat-HSP72 (lower panel) cells, showing the relative mean fluorescence intensity (rMFI). The black trace represents HSP72 expression, and the gray dotted trace denotes the isotype control. (c) Western blot of HSP72 expression in MOCK and HSP72-transfected Jurkat cells. **: $p < 0.01$.

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