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Original Article

HDAC6 deacetylates p53 at lysines 381/382 and differentially coordinates p53-induced apoptosis

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ABSTRACT

HDAC6-selective inhibitors represent promising new cancer therapeutic agents, but their precise mechanisms of action are not well understood. In particular, p53's role in HDAC6 inhibitor-induced effects has not been fully elucidated. In this study, we show that an HDAC6-selective inhibitor, A452, increased wild-type p53 levels by destabilizing MDM2, but decreased mutant p53 by inducing MDM2 and inhibiting Hsp90-mutant p53 complex formation. Interestingly, HDAC6 levels inversely correlated with p53 acetylation at lysines 381/382 associated with p53 functional activation. A452 blocked HDAC6 nuclear localization, resulting in increased levels of acetylated p53 at Lys381/382. HDAC6 bound to the C-terminal region of p53 via its deacetylase domain. A452 disrupted the HDAC6-Hsp90 chaperone machinery via Hsp90 acetylation and degradation. Furthermore, it chemosensitized cancer cells to the Hsp90 inhibitor 17-AAG. Overall, silencing of HDAC6 showed similar effects. These findings suggest that the anticancer action of HDAC6 inhibitors requires p53 and Hsp90 and targeting of HDAC6 may represent a new therapeutic strategy for cancers regardless of p53's mutation status.

Introduction

Selective Inhibition of histone deacetylase 6 (HDAC6) has emerged as an exciting and promising new approach for the development of cancer therapeutics. Because nonselective pan-HDAC inhibitors (HDACi) display adverse effects such as fatigue, nausea, vomiting, diarrhea, thrombocytopenia, and neutropenia, the discovery of isoform-specific HDACi may offer a therapeutic advantage by minimizing toxicity profiles. Among the existing HDACs, HDAC6 can influence a number of cancer-linked cellular pathways, making it a viable therapeutic target for cancer treatment. HDAC6 aberrant expression is correlated with malignant progression in various cancers including human breast cancer and ovarian cancer [1]. HDAC6 inhibition promotes α-tubulin acetylation, which enhances microtubule stability and reduces cell migration [2]. Through the ubiquitin-binding domain, HDAC6 in concert with p97/VCP, TRIM50, and p62 controls aggresome formation and autophagosome maturation for ubiquitin-selective quality-control autophagy [3,4]. Hyperacetylation of the chaperone heat shock protein 90 (Hsp90) in response to HDAC6 inhibition reduces its association with client proteins, resulting in ubiquitin-proteasome system-mediated degradation of many Hsp90 substrates [5]. Furthermore, HDAC6 inhibition can enhance the cytotoxicity of the proteasome inhibitor bortezomib [6–8].

The *TP53* gene, encoding the p53 transcription factor, is the most frequently mutated tumor-suppressor gene in human malignancies [9]. Anticancer chemotherapy outcomes can depend on the p53 status of tumors. Tumors with mutated (mut) or deleted p53 are less responsive to commonly used chemotherapeutics [10] and more malignant [11]. Since over 50% of human tumors have *TP53* mutations, 90% of which are missense point mutations in the p53 DNA-binding domain (DBD), therapeutic strategies that do not rely

Abbreviations: CHX, cycloheximide; CRC, colorectal cancer cells; DBD, DNA-binding domain; HDAC6, histone deacetylase 6; HDACi, histone deacetylase inhibitors; Hsp90, heat shock protein 90; KO, Knockout; MDM2, murine double minute-2; MDMX, murine double minute-X; MEF, mouse embryonic fibroblast; PARP, poly(ADP ribose) polymerase; SAHA, suberoylanilide hydroxamic acid; siRNA, small interfering RNA; shRNA, short hairpin RNA.

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on functional p53 are clinically preferable. However, because the mutp53 protein is retained and accumulates in tumors, destabilizing mutp53 may provide a novel therapeutic strategy of clinical significance. Thus, there is a need to develop an anticancer agent that is effective in both wild-type (wt) p53 and mutp53 harboring tumors.

To shed more light on this issue, we investigated the extent to which the anticancer effects of HDAC6-selective inhibition are influenced by p53 status using cancer cells expressing different p53 mutations. Here, we report anticancer mechanisms of action of the novel γ-lactam–based HDAC6 inhibitor A452. Cytotoxicity induced by HDAC6-selective inhibition is predominantly caused by its ability to strongly stabilize wtp53 and destabilize mutp53. These HDAC6 effects on p53 stability and activity are attributed to the HDAC6-mediated p53 deacetylation at lysines 381/382. Collectively, these data provide encouraging evidence for the feasibility of p53-targeted anticancer therapy using a new HDAC6-selective inhibitor.

Materials and methods

Experimental procedures

Small interfering RNA (siRNA) and short hairpin RNA (shRNA) transfection, immunoblot, immunofluorescence, immunohistochemistry, immunoprecipitation, subcellular fractionation, drug combination analysis, and Annexin V-PI assay were performed using standard methods. For other details, see Supplementary Materials and Methods.

Cell culture

Human cancer cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). All MEFs used were kindly provided by Patrick Matthias (Friedrich Miescher Institute), Tso-Pang Yao (Duke University), and Joo-Yong Lee (Chungnam National University). p53 null HCT116 cell line was generous gift of In-Chul Park (Korea Institute of Radiological & Medical Sciences) [12,13]. Some of CRC cell lines were generous gift of Jung Min Han (Yonsei University). Cells were cultured in medium (HyClone, Thermo Scientific Pierce, Rockford, IL, USA) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

Human colorectal cancer tissues

For 12 stage II colorectal cancer patients who had undergone 5-FU based adjuvant chemotherapy after radical resection, frozen tissues were obtained from malignant infiltrating tumors with paired normal colonic mucosa. This study was carried out according to the provisions of the Helsinki Declaration of 1975 and was reviewed and approved by the Institutional Review Board (IRB-3-2014-0287).

HDAC6 expression in TCGA colorectal carcinoma cohort

The level 3 normalized mRNA expression of HDAC6 was retrieved from the archive of The Cancer Genome Atlas (TCGA) via Broad GDAC Firehose (https://gdac.broadinstitute.org). The information of HDAC6 expression was acquired in 383 tumors and 51 normal adjacent tissues after filtering and compared.

Cell growth and viability assay

Cell growth and viability assays were performed according to the manufacturer's instructions (CCK-8 kit, CK04, Dojindo Molecular Technologies, INC. Rockville, MD, USA). To monitor cell growth and viability, cells were seeded in triplicate at $3–6 \times 10^3$ cells in 200 μl of medium in 96-well plates. The drugs were added at the indicated concentrations 24 h after seeding. After drug treatment, 20 μl of a water-soluble tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H* tetrazolium, monosodium salt (WST-8) reagent were added to the culture and reaction mixtures were incubated at 37 °C for 4 h. The absorbance readings for each well were carried out at 450 nm using the multimode microplate reader (Teckan, Maennedorf, Switzerland). Results are presented as the percent absorbance relative to control cultures and were generated from three independent experiments performed in triplicate.

Statistical analysis

All data are presented as the mean ± SD from three independent experiments. Statistical significance was determined by Student's *t* test of treated samples when compared to respective control, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results

HDAC6 inhibition differentially modulates p53 by upregulating wild-type and downregulating mutant p53

To investigate a potential interplay between p53 and HDAC6 at the transcriptional level, we determined the mRNA expression levels of p53 and its downstream target genes by real-time RT-PCR after A452 treatment. A452 is a γ-lactam-based HDAC6-selective hydroxamate inhibitor [14]. A452 did not affect p53 mRNA expression in HCT116 or HT29 colorectal cancer (CRC) cells, but upregulated p21 expression (Fig. S1A and B). Interestingly, murine double minute-2 (MDM2) and murine double minute-X (MDMX), which are key negative regulators of p53 [15], were upregulated by A452 in mutp53 HT29 cells. In contrast, MDM2 was upregulated by A452 but MDMX downregulated in wtp53 HCT116 cells. Furthermore, A452 caused induction of the downstream target genes Bax and Gadd45 in both CRC cells. However, p53 target genes including MDM2 remained unchanged by A452 in p53 null HCT116 cells (Fig. S1C).

To verify the mRNA expression data and to gain more insight into the p53-dependent and p53-independent effects of HDAC6-selective inhibition, we performed immunoblotting. A452 strongly upregulated wtp53 protein in HCT116 and RKO cells but downregulated mutp53 protein in HT29 and SW620 cells (Fig. 1). Consistent with the western blot, the altered levels of p53 by A452 was further confirmed by immunofluorescence (Fig. 3B–E). MDM2 decreased while MDMX remained unchanged by A452 in wtp53 HCT116 and RKO cells (Fig. 1B and C). Ubiquitination assays showed increased ubiquitination of MDM2 after A452 treatment in wtp53 HCT116 cells (Fig. S2A). Treatment with cycloheximide (CHX), a protein synthesis inhibitor, did not prevent the reduction of MDM2 level after A452 treatment (Fig. S3A), suggesting that A452 significantly shortens the half-life of MDM2 in wtp53 HCT116 cells. However, MDM2 increased while the ubiquitination level decreased without changes in MDMX in mutp53 HT29 and SW620 cells (Fig. 1B and D; Fig. S2B). Also, A452 significantly prolonged the half-life of MDM2 in mutp53 HT29 cells (Fig. S3B). Proteasome inhibition by MG132 completely rescues A452-mediated downregulation of mutp53, indicating that A452 regulates its stability on the post-transcriptional level (Fig. S3B). The altered levels of p53 and stable levels of MDMX by A452 were also confirmed in CHX chase experiments (Fig. S3). Consistent with the mRNA data, A452 increased the expressions of p53 target genes p21, Bax, and Bak and p53 transactivation activity in wtp53 HCT116 and RKO cells (Fig. S4A–C). The expression of the downstream p53 targets was slightly enhanced by A452, while A452 did not influence p53 transactivation activity in HT29 and SW620 cells harboring the R273H DBD p53 mutation (Fig. S4A and B). Similar to A452 treatment, the known HDAC6-selective inhibitor tubastatin A (Fig. S5) and HDAC6 knockdown by siRNA (Fig. 2C and D) showed similar effects, suggesting that this effect is due to HDAC6 inhibition. To further confirm this result, we tested the A452's effect on MDM2, MDMX and p53 expression in a panel of cancer cell lines possessing different p53 status levels. Similar results were also observed in other cancer cells (Fig. S6). In contrast, romidepsin, a specific inhibitor for HDAC1/2, did not alter the expression levels of p53, MDMX and p53 target genes in wtp53 HCT116 and mutp53 HT29 cells (Fig. S7). Romidepsin upregulated p21 whereas downregulated MDM2 in a p53-independent manner. Also, the level of HDAC6 protein was negatively correlated with that of wtp53 ($R^2 = -0.70$) while positively correlated with that of mutp53 ($R^2 = 0.69$) in CRC cells (Fig. S8). Overall, these results strongly suggest that mutp53 downregulation and wtp53 upregulation and altered levels of MDM2 by A452 are due to HDAC6-specific inhibition.