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Small molecules inhibiting the nuclear localization of YAP/TAZ for chemotherapeutics and chemosensitizers against breast cancers

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ABSTRACT

YAP and TAZ oncoproteins confer malignancy and drug resistance to various cancer types. We screened for small molecules that inhibit the nuclear localization of YAP/TAZ. Dasatinib, statins and pazopanib inhibited the nuclear localization and target gene expression of YAP and TAZ. All three drugs induced phosphorylation of YAP and TAZ, and pazopanib induced proteasomal degradation of YAP/TAZ. The sensitivities to these drugs are correlated with dependence on YAP/TAZ in breast cancer cell lines. Combinations of these compounds with each other or with other anti-cancer drugs efficiently reduced cell proliferation of YAP/TAZ-dependent breast cancer cells. These results suggest that these drugs can be therapeutics and chemosensitizers for YAP/TAZ-dependent breast cancers.

1. Introduction

YAP and TAZ are transcriptional co-activators involved in tissue growth and stem cell maintenance in normal tissue through binding to TEADs. They are regulated through phosphorylation by the Hippo pathway, leading to inhibition of the nuclear translocation and the proteasomal degradation [1]. The Hippo pathway is activated by cellular density [2–4], soluble factors via GPCR [5,6], and actin cytoskeleton organization [7,8]. YAP and TAZ activations are also implicated in the tumorigenesis and malignancy of various cancers including breast [9], colon [10], lung [11], liver [12], and mesothelioma [13]. TAZ is associated with the maintenance of breast cancer stem cells and drug resistance [14]. Therefore, YAP and TAZ play causative roles in carcinogenesis and cancer progression, and inactivation of YAP and TAZ by small molecules is a promising strategy for therapeutics of various cancers with their activation [15].

In drug discovery, one of the successful strategies is the exploitation of established drugs that have already been approved for treatment of other cancers or non-cancerous diseases (i.e., drug repositioning, drug repurposing, or indication switch). The major advantage of this approach is that the pharmacokinetic, pharmacodynamics and toxicity profiles of these drugs are well known, making their rapid shifts to clinical trials possible [16,17].

In this study, we screened for small molecules which inactivate YAP and TAZ from drugs with known targets for the drug repositioning against breast cancer. We found that dasatinib, statins, and pazopanib inhibited their nuclear localization and TEAD-dependent transcription, and induced YAP/TAZ phosphorylation. Pazopanib induced proteasomal degradation of YAP and TAZ. Furthermore, we explored the possibility of chemotherapy with them for breast cancer, and found that the sensitivities to these compounds are correlated with the dependence on YAP and TAZ in these cell lines. Combination of the YAP/TAZ inhibitors or of those with anti-cancer agents efficiently suppressed the breast cancer cell growth. Our findings thus opened the window for the application of dasatinib, statins, and pazopanib, clinically used drugs, for breast cancers with activation of YAP and TAZ.

Abbreviations: YAP, yes-associated protein; TAZ, transcriptional coactivator with PDZ-binding motif; TEAD, TEA domain family member; GPCR, G-protein coupled receptor; FDA, food and drug administration; NF2, neurofibromin 2; HMG-CoA, hydroxymethylglutaryl-coenzyme A; CTGF, connective tissue growth factor

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2. Materials and methods

2.1. Cell culture and treatments

MDA-MB-231, MDA-MB-453, HBC-4, HBC-5, MCF-7, BSY-1, ZR-75-1, and SKBR-3 breast cancer cell lines were maintained in RPMI-1640 containing 10% FBS and penicillin/streptomycin. HEK293 was maintained in Dulbecco's modified Eagle medium containing 10% FBS and penicillin/streptomycin. Dasatinib was purchased from JS Research Chemicals Trading Co. Fluvastatin, doxorubicin, and paclitaxel were purchased from Wako Pure Chemicals. Geranylgeranyl diphosphate (GGPP), farnesyl diphosphate (FPP), GGTI-286, and FTI-276 were purchased from Sigma. MG-132 was purchased from Merck Mllipore, and pazopanib from ChemieTek.

2.2. Antibodies

For immunofluorescence, 1/200 rabbit anti-YAP antibody (H-125, Santa Cruz), 1/400 rabbit anti-YAP/TAZ antibody (D24E4, Cell Signaling), 1/200 phalloidin-AlexaFluor 594 (Life Technologies), and 1/1000 anti-rabbit IgG-AlexaFluor 488 conjugate (Life Technologies) were used. For immunoblot, 1/3000 rabbit anti-YAP antibody (H-125, Santa Cruz), 1/3000 rabbit anti-TAZ antibody (#2149, Cell Signaling), 1/3000 rabbit anti-YAP/TAZ antibody (D24E4, Cell Signaling), 1/10,000 mouse anti-GAPDH antibody (6C5, Millipore), 1/5000 anti-mouse IgG-HRP (GE Healthcare), and 1/5000 anti-rabbit IgG-HRP (GE Healthcare), were used. Antibodies for Western blot were diluted in Can Get Signal reagents (Toyobo). Western blot using standard SDS–PAGE gel or gels containing Phos-tag-acrylamide (SuperSep Phos-tag, Wako) was performed as previously described [18].

2.3. Screening of the inhibitors inhibiting nuclear localization of YAP

MDA-MB-231 cells (10,000–15,000 cells) were inoculated in a μclear imaging plate (Corning) and 24 h later, 10 μM chemicals in a SCADS inhibitor kit (provided by the Screening Committee of Anticancer Drugs, Japan) was added and incubated for 6 h. Cells were fixed with 4% paraformaldehyde and immunostained with anti-YAP antibody as described below.

2.4. Immunofluorescence and imaging

Paraformaldehyde-fixed cells were permeabilized with 0.3% TritonX-100 in PBS and blocked with 3% FBS in PBST for 30 min. They were then incubated with anti-YAP antibody at 4 °C overnight and washed with PBS three times. Cells were incubated with anti-rabbit IgG-Alexa Fluor 488 for 1 h at room temperature, and washed with PBS three times. For confocal microscopy, cells were mounted in Prolong Gold reagent containing 10 μg/ml Hoechst 33342 (Life Technologies). When appropriate, cells were stained with phalloidin-Alexa Fluor 594 (Life Technologies) prior to mounting. Images were obtained with an FV1000-D confocal microscope equipped with a 40× objective lens using FV10-ASW software (Olympus). For screening of small molecules, PBS containing 10 μg/ml Hoechst 33342 was added and images were obtained by IN Cell Analyzer 2000 (GE Healthcare) using a 40× objective lens.

2.5. Reporter assay

8xGTIIC-luciferase was obtained from Addgene (Addgene #34615). MDA-MB-231 cells were transfected with 1.4 μg of 8xGTIIC-luciferase plasmid and 50 ng of pRL-CMV (Promega) by using lipofectamine 2000 (Life Technologies) or Viafect transfection reagent (Promega) as previously described [7]. After 24 h, cells were treated with chemicals for 18 h. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

2.6. Quantitative real time PCR

Total RNA was prepared with ISOGEN (Nippon Gene) and cDNA was synthesized with ReverTra Ace qPCR RT master mix with gDNA remover (TOYOBO). Real time PCR was performed with QuantiTect SYBR Green PCR Master Mix (Qiagen) using the Eco Real Time PCR system (Illumina). The sequences of PCR primers for *CTGF* and *GAPDH* were as follows; CTGF-F: AGGAGTGGGTGTGTGACGA, CTGF-R: CCAGGCAGTTGGCTCTAATC, GAPDH-F: AGCCACATCGCTCAGACAC, GAPDH-R: GCCCAATACGACCAAATCC.

2.7. RNAi

siRNA transfection was performed using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instructions. The siRNA sequence is listed as followed; siYAP: GACAUCUUCUGGUCAGAGAUU, siTAZ: ACGUUGACUUAGGAACUUUUU [14].

2.8. MTT assay

MTT assay was performed as previously described [18]. 3000–10,000 cells suspended in RPMI-1640 containing 1% FBS were seeded on 96 well plates. Fifteen μl of medium containing drugs was added, and cells were incubated for 4 days.

2.9. Colony formation assay

MDA-MB-231 or MCF-7 cells (1000–2000 cells per well) were seeded on 24 well plates and treated with inhibitors for 10 days. Cells were fixed with 4% formaldehyde and stained with 0.5% crystal violet.

3. Results

3.1. Dasatinib, fluvastatin, and pazopanib inhibit the function of YAP/TAZ transcriptional co-activator

To identify drugs targeting YAP and TAZ, we performed image-based screening for small molecules which inhibit their nuclear localization using MDA-MB-231 breast cancer cell line. MDA-MB-231 harbors homozygous mutation in *NF2*, which positively regulates the Hippo pathway; therefore, YAP and TAZ are constitutively activated in this cell line [19]. We screened about 400 chemicals with known targets from SCADS inhibitor kit (provided by the Screening Committee of Anticancer Drugs, Japan) which consists of classical anti-cancer agents, kinase inhibitors, metabolic pathway inhibitors, and signaling pathway inhibitors, including FDA-approved drugs [20]. MDA-MB-231 cells were treated with chemicals and nuclear localization of YAP was evaluated by immunofluorescence. Chemicals which induced nuclear exclusion of YAP were treated as positive. We found that thiazovivin, dasatinib, lovastatin, cucurbitacin I, and pazopanib inhibited the nuclear localization of YAP (Fig. 1A). Among them, dasatinib, statins, and pazopanib are approved as clinically used drugs, and we therefore analyzed them further. We found that dasatinib, fluvastatin, and pazopanib inhibited nuclear localization of YAP and TAZ in the nanomolar to micromolar range, although the effect of pazopanib on the nuclear localization of YAP/TAZ was relatively weak compared to the other two drugs (Fig. 1B). They also