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Reversible induction of PARP1 degradation by p53-inducible cis-imidazoline compounds

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ABSTRACT

PARP1 is an important enzyme involved in various patho-physiological phenomena such as ischemia/reperfusion (I/R) injury, which occurs when blood flow is restored after cerebral infarction, myocardial infarction and transplantation of various organs. I/R-induced PARP1 over-activation is mediated by production of reactive oxygen species and is involved in NF-κB transactivation. For these reasons, PARP1 is an attractive target for strategies to protect against I/R injury. We previously reported that an MDM2 inhibitor Nutlin3a, a cis-imidazoline compound, induces PARP1 degradation in a p53 and proteasome-dependent manner. In this study, we evaluated the effect of Nutlin3a analogs, Nutlin3b and Caylin2, on PARP1 degradation. Like Nutlin3a, Caylin2, but not Nutlin3b, induced PARP1 degradation in both 3T3-L1 and 3T3-F442A. This result occurred almost in parallel with p53 accumulation. Furthermore Caylin2-induced PARP1 degradation was not observed in p53 deficient mouse embryonic fibroblasts or in the presence of the proteasome inhibitor MG132. These results suggest that Caylin2 induces PARP1 degradation by the same mechanism as Nutlin3a. Finally, we showed that Nutlin3a or Caylin2 treatment induces reversible PARP1 down-regulation without an inflammatory response. For protection against I/R injury, our results support the usability of the p53 inducible cis-imidazoline compounds, Nutlin3a and its analogs, as PARP1 inhibitors.

1. Introduction

Poly(ADP-ribosyl)ation, which is the post-translational protein modification, is involved in cell replication, DNA repair, cell death, and inflammation [1,2]. PARP1 is the most abundant PARP family member in cells, and is dramatically activated by DNA breaks. Therefore, massive DNA damage induces over-activation of PARP1, and then decreases ATP levels via over-consumption of cellular NAD^+, which is required for the ATP production in glycolysis and TCA cycle pathways. It has been also reported that PARP1 over-activation is involved in ischemia/reperfusion (I/R) injury, which occurs during the restoration of blood flow after cerebral infarction, myocardial infarction and organ transplantation [3–5]. For these reasons, PARP1 is an attractive target for protection against I/R injury [6].

We previously reported that Nutlin3a, an MDM2 ubiquitin ligase antagonist, induces p53 and proteasome-dependent PARP1 protein degradation [7]. It has been thought that Nutlin3a is a candidate for anti-tumor drugs, because MDM2 inhibition by Nutlin3a induces p53 stabilization, followed by p53-dependent apoptosis in tumor cells [8]. The discovery of Nutlin3a-induced PARP1 degradation prompted the use of Nutlin3a as a PARP1 inhibitor. Furthermore, considering that p53 has the potential to up-regulate anti-oxidant and anti-inflammatory genes [9–11], Nutlin3a may be a potent anti-I/R drug that has multiple points of action. However, the Nutlin3a pharmacophore that induce PARP1 protein degradation has not been identified. In the present study, to clarify whether Nutlin3a analogs were also able to induce PARP1 protein degradation in a manner similar to Nutlin3a, we examined the effect on PARP1 degradation by the commercially available Nutlin3a enantiomer, Nutlin3b [12,13], and by the Nutlin3a derivative, Caylin2 [14]. Furthermore, by using compounds possessing PARP1 degradation activity, we evaluated the reversibility of PARP1 degradation and the effect on anti-inflammatory IL6 gene expression.

2. Materials and methods

2.1. Cell culture and drugs

Mouse fibroblast 3T3-L1 and 3T3-F442A cell lines were purchased from the RIKEN Bioresource Center (Japan) and the European Collection of Animal Cell Cultures (UK), respectively. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, low glucose) (WAKO, Japan) with 10% fetal calf serum

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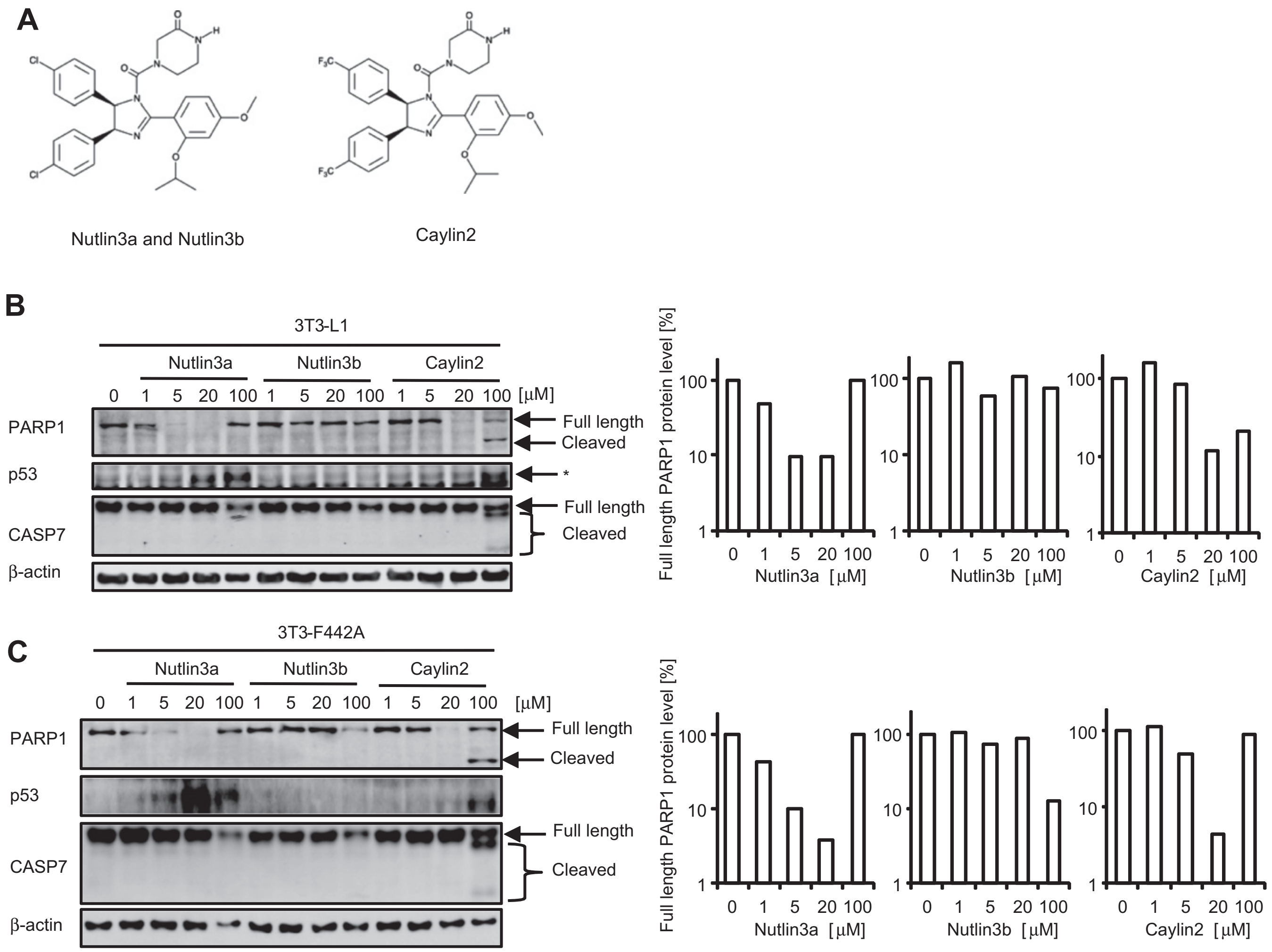

Fig. 1. Caylin2 but not Nutlin3b decreases in PARP1 protein levels in mouse fibroblasts. (A) Structures of Nutlin3a, Nutlin3b, and Caylin2. Mouse fibroblast 3T3-L1 (B) or 3T3-F442A (C) were treated with the indicated concentrations of Nutlin3a, Nutlin3b or Caylin2 for 8 h. The cell lysates were analyzed by Western blotting using the indicated antibodies (left pannel). Quantitative data are shown (right panel). In the p53 panel, the arrow and asterisk show the p53 and nonspecific bands, respectively. All experiments were performed at least three times, and representative data is shown.

and 1% penicillin/streptomycin (Sigma). p53+/+ or −/− MEFs were prepared as described previously [7]. The established MEFs were maintained in DMEM (high glucose) with 10% FCS, 0.1 mM 2-mercaptoethanol, and 1% penicillin/streptomycin. The proteasome inhibitor MG132 was purchased from WAKO (Japan). Nutlin3a, Nutlin3b, and Caylin2 were supplied by Cayman (USA).

2.2. Western blotting

Cell preparation and Western blotting were performed as described previously [7]. As primary antibodies, anti-PARP1 (clone C-2-10, WAKO, Japan), anti-p53 (clone Ab-1, Calbiochem, USA), anti-β actin (clone AC-15, SIGMA, USA), or anti-CASP7 (clone 1F3, MBL, Japan) antibodies were used. For secondary antibodies, horseradish peroxidase-conjugated F(ab′)$_2$ fragment of goat anti-mouse IgG or anti-rabbit IgG (Jackson Immunoresearch, USA) were used. The specific proteins were visualized with ImmunoStar LD reagent (WAKO, Japan) and LAS3000 (Fuji Film, Japan), and the data were analyzed using MultiGauge software (Fuji Film, Japan).

2.3. RNA purification and RT-PCR

RNA purification and RT-PCR were performed using RNAiso PLUS, FastPure RNA kit, PrimeScript Reverse Transcriptase and random hexamers (all from TaKaRa, Japan) as described previously [7]. The PCR was performed using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, USA) and primers for *TNFα* (forward, 5′-CCCTCACACTCAGATCATCTTCTC-3′; reverse, 5′-GCCTTGTCCCTTGAAGAGAACC-3′) *IL6* (forward, 5′-GCCTTCCCTACTTCACAAGTCC-3′; reverse, 5′-CAGAATTGCCATTGCACAAC-3′), or *TBP* (forward, 5′-CAGTACAGCAATCAACATCTCAGC-3′; reverse, 5′-CAAGTTTACAGCCAAG-ATTCACG-3′) as follows: initiation step, at 94 °C for 1 min; amplification step, at 94 °C for 1 min, at 60 °C for 15 s, at 68 °C for 15 s; termination step, 68 °C 15 s. PCR products were subjected to 1.8% agarose gel electrophoresis, stained with ethidium bromide, and visualized with LAS3000. The data was analyzed using MultiGauge software (Fuji Film, Japan).

3. Results

3.1. Caylin2. but not Nutlin3b induces a decrease in PARP1 protein levels in mouse fibroblast cell lines

Although we previously reported that Nutlin3a induces PARP1 protein degradation, we did not address whether Nutlin3a analogs also have the potential to induce PARP1 degradation [7]. Here, we investigated the inducibility of PARP1 degradation by two such analogs, Nutlin3b and Caylin2 in mouse fibroblast cell lines