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Anticancer effects of the p53 activator nutlin-3 in Ewing's sarcoma cells

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ABSTRACT

Mutation of p53 is rare in Ewing's sarcoma (ES), suggesting that targeting and activation of wild-type p53 may be an effective therapeutic strategy for ES. The recently developed small-molecule MDM2 inhibitor nutlin-3 restores wild-type p53 function, resulting in the inhibition of cancer cell growth and the induction of apoptosis. In the present study, we explored the responsiveness of ES cell lines with wild-type or mutated p53 to nutlin-3. We found that treatment with nutlin-3 increased p53 level and induced p53 target gene expression (MDM2, p21, PUMA) in ES cells with wild-type p53, but not in ES cells with mutated p53. Consistently, nutlin-3 elicited apoptosis only in wild-type p53 cells, as assessed by caspase-3 activity assay and flow cytometric analyses of mitochondrial depolarisation and DNA fragmentation. In addition, we found nutlin-3 to evoke cellular senescence, indicating that nutlin-3 induces pleiotropic anticancer effects in ES. Furthermore, combined treatment with nutlin-3 and an inhibitor of NF-κB produced synergistic antineoplastic activity in ES cells. Our findings suggest that the direct activation of p53 by nutlin-3 treatment may be a useful new therapeutic approach for patients with ES.

1. Introduction

The tumour suppressor protein p53 protects cells from neoplastic transformation by mediating cell cycle arrest and apoptosis.[1] The activities of p53 are under control of MDM2; in unstressed cells, MDM2 interacts with p53, thereby inhibiting its functions. Stress signals, such as DNA damage and oncogene activation, cause the dissociation of the p53–MDM2 complex, unchaining the antitumour activities of p53.

p53 is the most frequently inactivated protein in human malignancies. Its inactivation may arise from gene mutations: about 50% of human tumours bear alterations in the p53 gene.[2] But it may as well arise from a dysfunctional p53 signalling pathway; in this case, p53 function is disabled in the absence of mutations: of the 50% of human cancers with wild-type (wt-) p53, many have impaired p53 functions as a result of increased MDM2 levels.[3] Thus, rescue of p53 activities by preventing the interaction of p53 with MDM2 is an appealing approach for the treatment of wt-p53 tumours.

Molecules blocking the p53–MDM2 interaction have indeed been found to restore p53 function and to affect cancers with wt-p53.[4] Of them, the imidazoline inhibitor nutlin-3 was the first shown to have antineoplastic activity *in vivo*.[5] It has subsequently been demonstrated to be effective in a number of tumour models[6], and, importantly, it has been found to activate p53 without inducing DNA damage.[4] One of the nutlins, RG7112, has entered clinical trials with patients suffering from advanced solid tumours and haematologic malignancies.[7,8]

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Nutlin-3 may be an even more suitable agent for the treatment of paediatric cancers. While 50% of adult patients have tumours with mutated (mt-) p53 and are, thus, unlikely to benefit from nutlin-3 therapy, alterations in the p53 gene are much less common in childhood malignancies. For instance, among children with Ewing's sarcoma (ES), only about 10% have been found with p53 alterations.[9,10]

ES is the second most frequent primary bone tumour in childhood and adolescence. It is an aggressive malignancy, and before the era of chemotherapy, not more than 10% of ES patients survived.[11] The introduction of intensive multimodal treatment with combination chemotherapy, surgery and radiotherapy has improved the disease-free survival of patients with localised disease to about 70%. However, of the approximately 25% of patients with primary metastatic ES, only 10–40% survive.[12] Furthermore, patients who experience disease relapse have an extremely dismal prognosis, with a survival probability of less than 20%.[13] The therapeutic outcome of disseminated or recurrent ES, thus, is still unsatisfactory, and more effective treatment strategies are necessary to increase survival in these patients.

Nutlin-3 has already been shown to exert anticancer effects in childhood tumour models with wt-p53, e.g. in neuroblastoma[14–17], in retinoblastoma[18,19], in acute lymphoblastic leukaemia[20] and in rhabdomyosarcoma.[16,21] However, it has not yet been tested for antineoplastic activity against ES, although those 90% of ES patients with wt-p53 are potentially amenable to nutlin-3 treatment. Therefore, we investigated the effects of nutlin-3 in cultured ES cell lines, and we found it to be effective in inducing cell death and cellular senescence.

2. Materials and methods

2.1. Cell lines

WE-68, VH-64 and SK-ES-1 cells were provided by Dr. F. van Valen (Münster, Germany) and CADO-ES-1 cells were obtained from the DSMZ (Braunschweig, Germany). Cells were maintained in RPMI medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulphate (PAA, Cölbe, Germany). They were cultivated in collagen-coated (5 μg/cm^2; Roche, Mannheim, Germany) tissue culture flasks at 37 °C in a humidified 5% CO_2 incubator and routinely passaged when 90% confluent. Cell viability was determined by the trypan blue exclusion test. Cells were regularly inspected to be free of mycoplasma with the PCR mycoplasma detection kit from Applichem (Darmstadt, Germany).

2.2. Treatment of cells

Cells were seeded in collagen-coated 6-well tissue culture plates at 150,000 cells/well and treated with racemic nutlin-3 (Alexis, Grünberg, Germany) for 24 h (immunoblotting, quantitative PCR, caspase-3 assay), 48 h (flow cytometric analyses), 72 h (cell count) or 96 h (cellular senescence). To inhibit NF-κB activation, the NF-κB inhibitor caffeic acid phenethyl ester (CAPE; Alexis) was applied 1 h before administration of nutlin-3.

2.3. Immunoblotting

After harvesting, cells were lysed on ice for 15 min in RIPA buffer supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany) followed by brief sonification. Protein concentration was assayed using bicinchoninic acid (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. 5 μg (GAPDH) or 120 μg (p53) of total cellular protein per lane was separated by standard SDS–PAGE on 10% gel and electrophoretically transferred to nitrocellulose membrane (Whatman, Dassel, Germany). After blocking, p53 was immunodetected using mouse anti-p53 DO-2 monoclonal antibody (dilution 1:500; sc-53394, Santa Cruz Biotechnology, Heidelberg, Germany). Equal loading of protein was verified by the detection of GAPDH using mouse anti-GAPDH monoclonal antibody (dilution 1:10,000; Biodesign International, Saco, ME, USA). Peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (dilution 1:25,000; Dianova, Hamburg, Germany) was used as secondary antibodies followed by enhanced chemiluminescence detection (GE Healthcare, Freiburg, Germany) of specific signals.

2.4. Quantitative real-time RT-PCR

Total RNA was isolated using a Peqgold Total RNA Kit including DNase digestion (Peqlab, Erlangen, Germany). RNA was transcribed into cDNA using Omniscript (Qiagen, Hilden, Germany). Quantitative PCR for MDM2, p21 and PUMA was performed using the 7900HT Fast Real-Time PCR system (Applied Biosystems, Darmstadt, Germany). Expression levels were normalised to β-2-microglobulin. Reactions were done in duplicate using Applied Biosystems Taqman Gene Expression Assays (MDM2: Hs99999008_m1; p21: Hs00355782_m1; PUMA: Hs00248075_m1; β-2-microglobulin: Hs00187842_m1) and Universal PCR Master Mix. All procedures were carried out according to the manufacturers' protocols. The relative MDM2, p21 and PUMA expression was calculated by the $2^{(-\Delta\Delta Ct)}$ method.[22]

2.5. Viable cell count

Cells were seeded in duplicate and, after harvesting, counted under a microscope, and cell viability was assessed by trypan blue dye exclusion.

2.6. Flow cytometric analysis of cell death

Cell death was assessed by determining the integrity of the cell membrane by flow cytometric analysis of propidium iodide (PI; Sigma, Deisenhofen, Germany) uptake. After harvesting, cells were incubated for 5 min in 2 μg/ml PI in PBS at 4 °C in the dark and PI uptake was measured on a BD (Heidelberg, Germany) FACSCanto II. 10,000 cells were analysed in each sample; data were gated to exclude debris. The results from the CAPE assay were analysed by the combination index (CI) method according to Chou and Talalay[23] using Calcusyn software from Biosoft (Cambridge, UK). CI values of <1, =1 and >1 indicate synergism, additivism and antagonism, respectively.