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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.

2.7. Flow cytometric analysis of mitochondrial transmembrane potential ($\Delta\psi_m$)

$\Delta\psi_m$ was determined by assessing the accumulation of 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] in the mitochondrial matrix. Before harvesting, cells were incubated with 50 nM $DiOC_6(3)$ (Molecular Probes, Eugene, OR, USA) at 37 °C for 30 min. After washing, 10,000 cells were analysed using the FACSCanto II. Data were gated to exclude debris.

2.8. Flow cytometric analysis of DNA content

To measure DNA content, ethanol-fixed cells were analysed for PI incorporation into DNA. After harvesting, cells were washed twice with PBS and fixed in 70% ethanol at –20 °C over night. After centrifugation, cells were resuspended in PBS containing 1% glucose, 50 μg/ml RNase A (Roche) and 50 μg/ml PI and incubated in the dark at room temperature for 30 min. Flow cytometric analysis was performed on the FACSCanto II. 20,000 cells were analysed in each sample; data were gated to exclude debris. Sub-G_1 phase cells were calculated from the DNA content histograms.

2.9. Caspase-3 activity

Caspase-3 activity was measured using the fluorogenic substrate Ac-DEVD-AMC (Bachem, Weil am Rhein, Germany). After harvesting, cells were lysed in 10 mM Tris–HCl, 10 mM $NaH_2PO_4/NaHPO_4$ (pH 7.5), 130 mM NaCl, 1% Triton X-100, and 10 mM $Na_4P_2O_7$ and then incubated with 20 mM Hepes (pH 7.5), 10% glycerol, 2 mM DTT, and 25 μg/ml Ac-DEVD-AMC at 37 °C for 2 h. The release of AMC was measured on

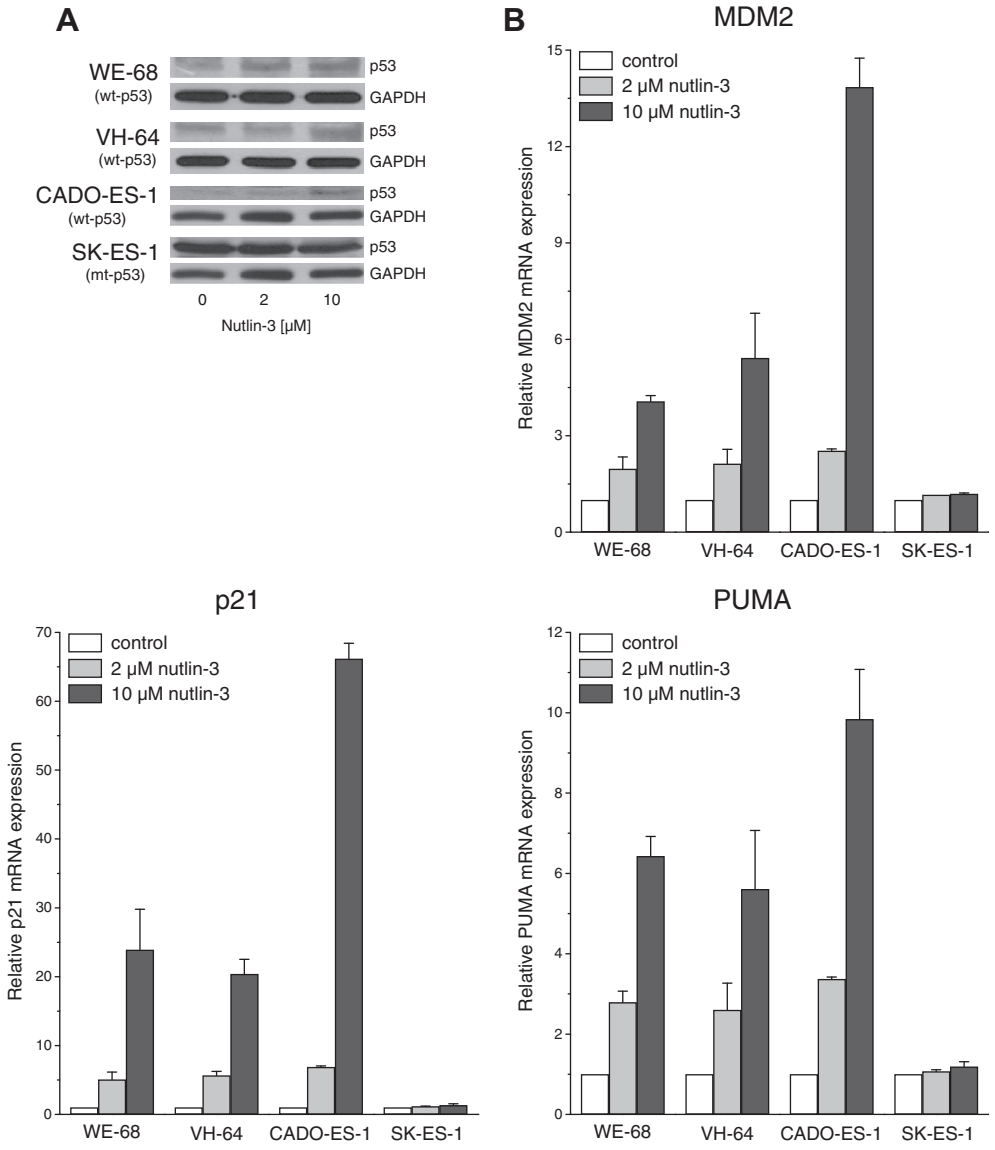

Fig. 1 – Nutlin-3 increases p53 level and induces expression of p53 target genes in ES cells with wt-p53. Cells were exposed to nutlin-3 for 24 h. (A) The abundance of p53 was determined by immunoblotting using the anti-p53 DO-2 antibody. (B) MDM2, p21 and PUMA mRNA expression levels were determined by real-time RT-PCR and normalised to β-2-microglobulin mRNA levels. Means ± SEM of each two separate measurements are shown.

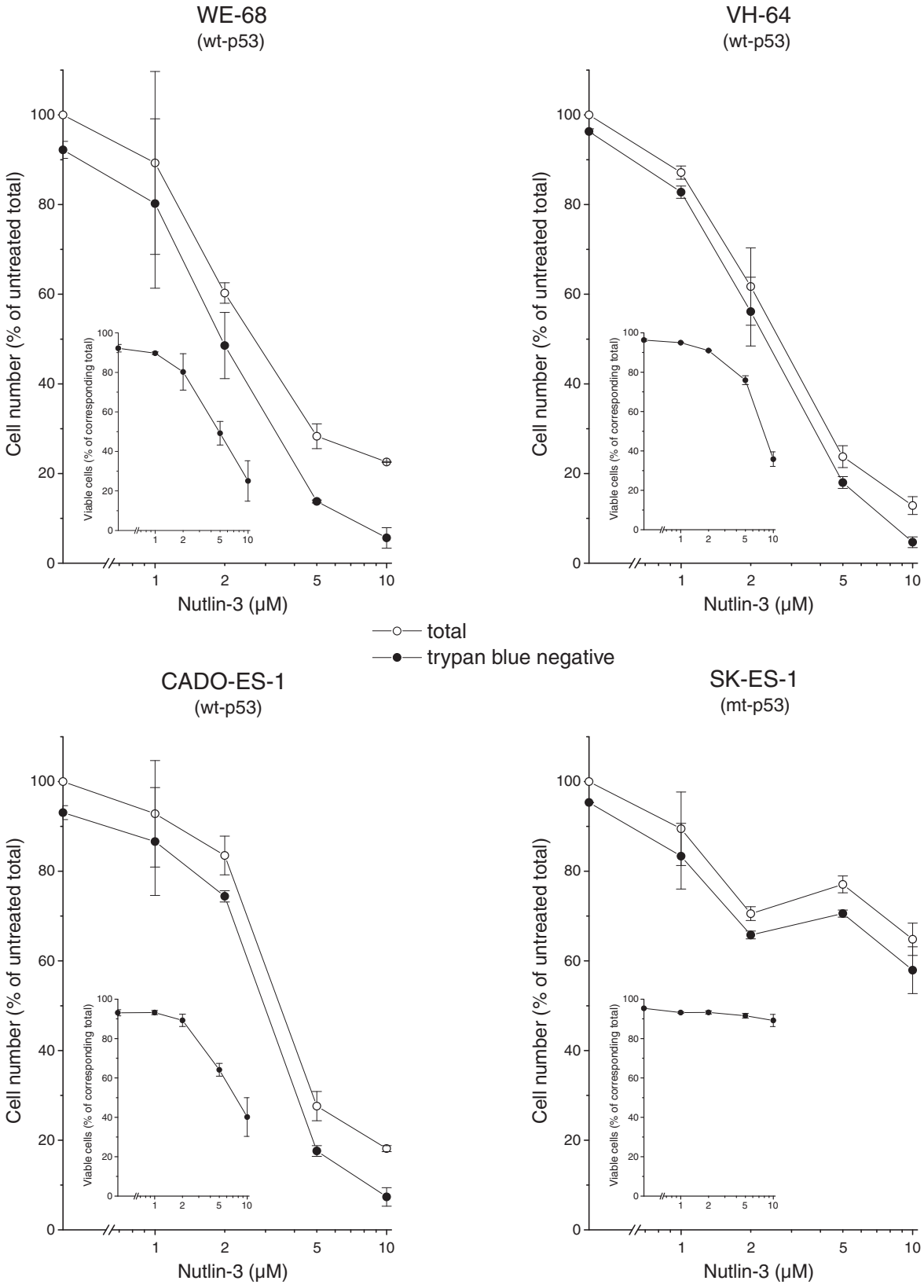

Fig. 2 – Nutlin-3 inhibits cell growth and induces cell death in ES cells. Exponentially growing cells were exposed to nutlin-3 for 72 h and cell growth and viability were determined by trypan blue exclusion cell count. The insets show the percentage of viable cells at each drug concentration. Means ± SEM of each two separate measurements are shown.

a BMG Labtech (Offenburg, Germany) FLUOstar Omega using an excitation/emission wavelength of 355/460 nm. Relative caspase-3 activities were calculated as a ratio of emission of treated cells to untreated cells.

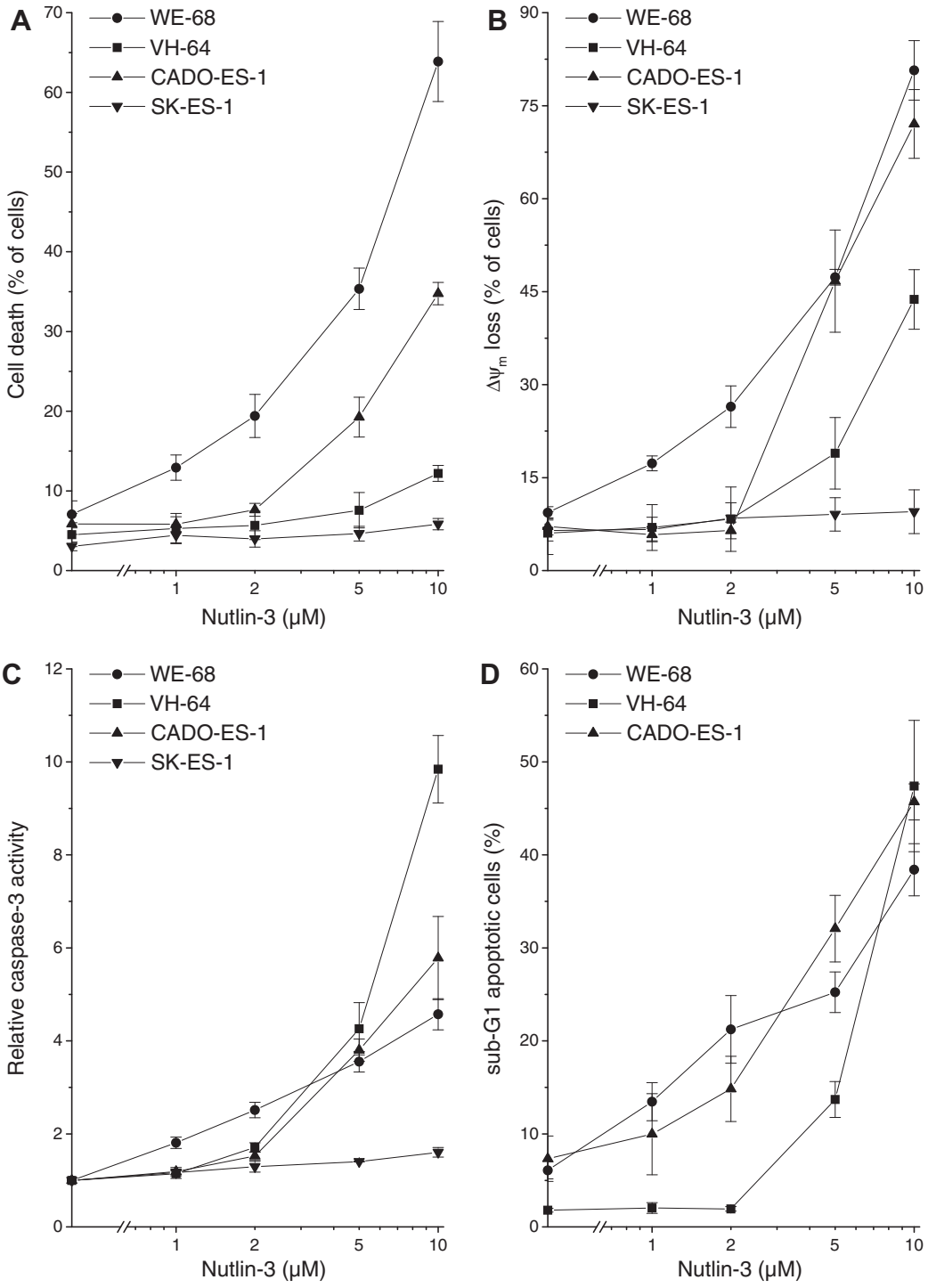

Fig. 3 – Nutlin-3 induces apoptosis in ES cells with wt-p53. Cells were exposed to nutlin-3 for 24 h (C) or 48 h (A, B, D). (A) Cell death was determined by flow cytometric analysis of PI uptake. (B) $\Delta\psi_m$ was determined by flow cytometric analysis of $DiOC_6(3)$ staining. (C) Caspase-3 activity was determined using the fluorogenic substrate Ac-DEVD-AFC; relative caspase-3 activities are the ratio of treated cells to untreated cells. (D) DNA fragmentation was determined by flow cytometric cell cycle analysis; apoptotic cells were detected as sub-G1 fraction. Means ± SEM of each three separate measurements are shown.

2.10. *Cellular senescence assay*

Cellular senescence was determined by assessing senescence-associated β-galactosidase (SA-β-Gal) activity at pH 6.0. At the end of the treatment period, cells were washed with PBS, fixed for 5 min with 1% glutaraldehyde, washed with PBS, and incubated at 37 °C for 18 h in fresh staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [Peqlab], 0.5 mM $K_3Fe[CN]_6$, 0.5 mM $K_4Fe[CN]_6$, 150 mM Nacl, 2 mM $MgCl_2$ in 40 mM citric acid/sodium phos-

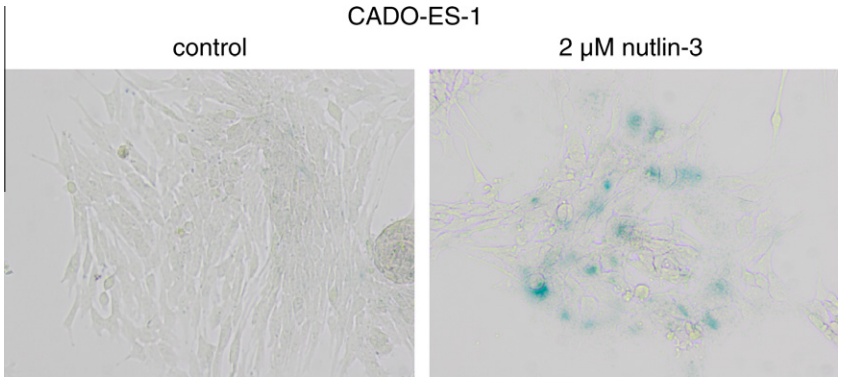

Fig. 4 – Nutlin-3 induces cellular senescence in CADO-ES-1 cells. After a 96-h exposure to nutlin-3 or vehicle control, cellular senescence was detected by staining for SA-β-Gal activity.

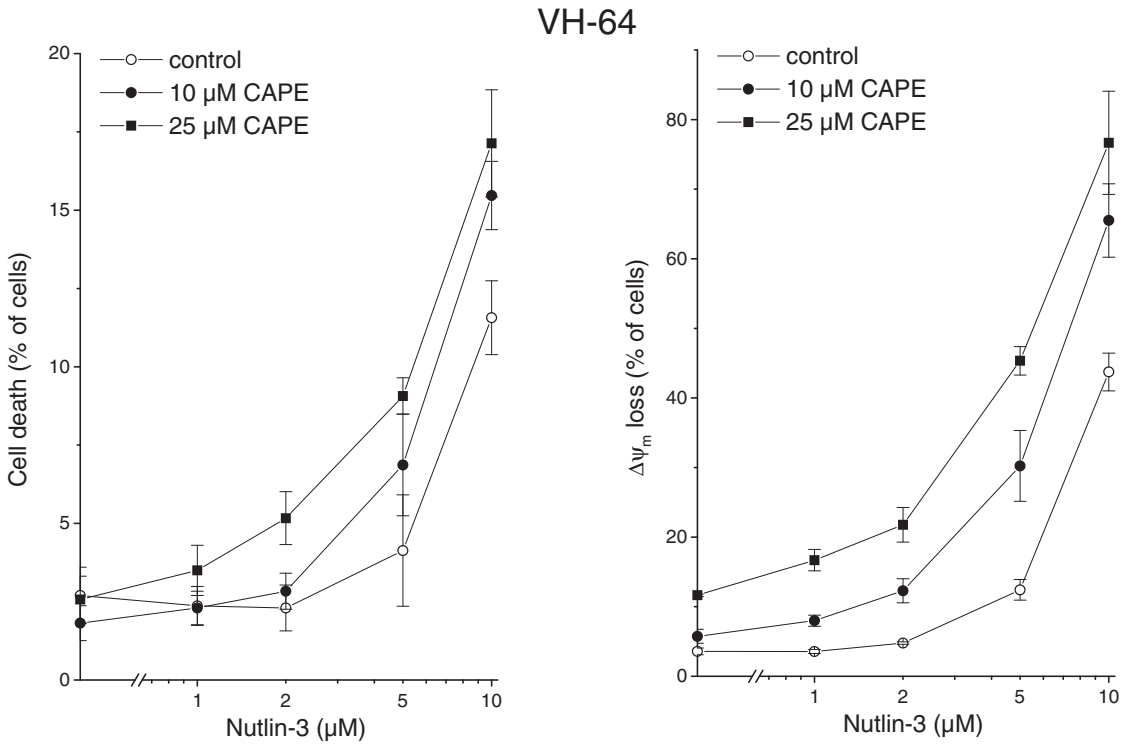

Fig. 5 – Nutlin-3 and the NF-κB inhibitor CAPE cooperate in inducing cell death and $\Delta\psi_m$ loss in VH-64 cells. One hour after administration of CAPE, cells were exposed to nutlin-3 for another 48 h. Cell death and $\Delta\psi_m$ were determined by flow cytometric analyses of PI uptake and $DiOC_6(3)$ staining, respectively. Means ± SEM of each three separate measurements are shown.

phate, pH 6.0). After washing with PBS, cells were viewed with an Olympus (Hamburg, Germany) CKX41 phase contrast microscope.

3. Results

3.1. *Nutlin-3-induced effects on p53 and p53 targets*

To establish that the action of nutlin-3 is dependent on the p53 gene status in ES cells, we studied its effects in three ES cell lines with wt-p53 (WE-68, VH-64 and CADO-ES-1) and in one ES cell line with mt-p53 (SK-ES-1).[9,24] Initially, we assessed the impact of nutlin-3 on p53 abundance. Immunoblot detection revealed a rise of p53 after nutlin-3 exposure in the ES cell lines with wt-p53 (Fig. 1A). Consistent with the high expression of mt-p53 observed in a wide range of tumours[25], mt-p53 SK-ES-1 cells displayed a much stronger constitutive expression of p53, which, however, was not enhanced by nutlin-3 treatment. To confirm the p53-activating effect of nutlin-3, we determined the gene expression of three key transcriptional targets of p53[26] – MDM2, p21 and PUMA – by real-time RT-PCR. As shown in Fig. 1B, nutlin-3 induced concentration-dependent increases in mRNA levels in all three cases in the wt-p53 cell lines, but not in the mt-p53 one.

3.2. Nutlin-3-induced antineoplastic effects in ES cells

To investigate the effect of nutlin-3 on cell growth and cell death, we conducted differential cell counts of total and viable cells. Fig. 2 demonstrates that nutlin-3 affected cell growth in a dose-dependent manner. At 72 h after treatment with 10 μM nutlin-3, the total number of cells (open symbols) was reduced by 77–87% in the wt-p53 cells and by 35% in the mt-p53 cells. More significantly, the number of viable cells (solid symbols), as determined by trypan blue exclusion cell count, was decreased by 93–95% in the wt-p53 cells and by 42% in the mt-p53 cells. The calculation of fractions of viable cells at each drug concentration revealed a decline in cell viability of 60–75% in the wt-p53 cells and of 11% in the mt-53 cells (inset diagrams). These findings show that nutlin-3 inhibits cell growth in both wt-p53 cells and – to a lesser extent – mt-p53 cells, but induces significant cell death only in wt-p53 cells.

To gain further insight into nutlin-3-elicited cell death and, more specifically, apoptosis, we analysed the effects of nutlin-3 by a number of read-outs. To begin with, we assessed cell death by flow cytometric analysis of PI uptake. Treatment with nutlin-3 resulted in a concentration-dependent induction of cell death in cells with wt-p53, but not in cells with mt-p53 (Fig. 3A). p53 is a potent inducer of apoptosis and as such it predominantly triggers the mitochondrial pathway of apoptosis[26]. We, therefore, examined whether nutlin-3-mediated cell death involved apoptosis. The same was evaluated by measuring $\Delta\psi_m$ dissipation, caspase-3 activity and DNA fragmentation. First, we determined $\Delta\psi_m$ loss by flow cytometric analysis of $DiOC_6(3)$ staining. As presented in Fig. 3B, the results reflect those of the cell death assay: nutlin-3 induced decay of $\Delta\psi_m$ in the wt-p53 cells, but not in the mt-p53 cells. Second, we measured caspase-3 activity. In consistence with the other read-outs, nutlin-3 caused caspase-3 activation in the wt-p53 cells, but not in the mt-p53 cells (Fig. 3C). Third, we assessed cells harbouring wt-p53 for apoptosis by staining the nuclei of ethanol-fixed cells with PI and determining the DNA content by flow cytometry. Fig. 3D demonstrates that nutlin-3 promoted DNA fragmentation in a dose-dependent fashion in the three wt-p53 cell lines.

3.3. Induction of cellular senescence by nutlin-3

In addition to apoptosis, p53 can also trigger cellular senescence.[1] Accordingly, nutlin-3 has been reported to induce a senescence response.[15] We thus addressed the question of whether nutlin-3 could promote cellular senescence in ES cells. After a 4-d exposure to nutlin-3 at a dose (2 μM) that had hardly exerted any apoptosis-inducing effect, CADO-ES-1 cells were stained for SA-β-Gal activity, the most widely used marker for cellular senescence. Nutlin-3-treated cells showed clear signs of senescence, whereas untreated cells did not (Fig. 4).

3.4. Synergistic effect of nutlin-3 with the NF-κB inhibitor CAPE

The combination of nutlin-3 with other anticancer agents has been shown *in vitro* to have synergistic effects on tumour cells.[4]

Table 1 – Combination index values for CAPE plus nutlin-3 in VH-64 cells.

CAPE (μM)	Nutlin-3 (μM)	CI
10	1	1.251
10	2	1.372
10	5	0.761
10	10	0.419
25	1	0.829
25	2	0.598
25	5	0.514
25	10	0.356

Based on data from Fig. 5, CI values were calculated using the Chou–Talalay method.

However, though the simultaneous targeting of p53 and NF-κB is considered a promising antineoplastic strategy[27], a possible favourable interaction between nutlin-3 and NF-κB inhibitors has not yet been studied. To explore this issue, we examined whether nutlin-3 and the NF-κB inhibitor CAPE would cooperate in exerting antitumour activity against wt-p53 ES cells. For this purpose, VH-64 cells were chosen because they had exhibited the weakest response to nutlin-3 treatment, as judged by PI uptake and $DiOC_6(3)$ staining analyses (see Fig. 3A and B). As shown in Fig. 5, nutlin-3 and CAPE cooperated both in eliciting cell death and mitochondrial depolarisation. To test for synergy, we analysed the cell death data by the CI method ($CI < 1$ is indicative for a synergistic interaction[23]). The calculated CI values indicated synergism for the combinations of 10 μM CAPE with 5 or 10 μM nutlin-3 and for 25 μM CAPE with nutlin-3 at all concentrations (Table 1).

4. Discussion

The direct and specific targeting of p53 provides an enticing therapeutic option for ES in view of the low prevalence of p53 mutations in this type of tumour. Our study is the first to explore the pharmacological activation of p53 as a novel approach for the treatment of ES.

In this study, we have found that treatment with nutlin-3 increased p53 protein and induced expression of p53 target genes in ES cells with wt-p53, but not in ES cells with mt-p53. We have as well found that nutlin-3 elicited significant cell death only in wt-p53 cells. These findings are consistent with the prevailing notion that the functional effects of nutlin-3 treatment primarily depend on the presence of wt-p53.[4] Yet, we have also noted that nutlin-3 reduced cell growth in mt-p53 cells. This may be explained by residual activity of the point-mutated (C176F)[9] p53 in SK-ES-1 cells or by p53-independent effects of nutlin-3. The former explanation is disfavoured by the lack of effect of nutlin-3 on p53 abundance and p53 target gene expression in SK-ES-1 cells. The latter, however, is in accordance with recent articles in which nutlin-3 was documented to reduce cell viability in mt-p53 cells[15], to induce cell cycle arrest in p53-deficient cells[28], and to enhance chemotherapy-mediated apoptosis in mt-p53 cells.[29] p53-independent nutlin-3 effects were attributed to the nutlin-3-mediated activation of other MDM2 binding partners, i.e. the transcription factor E2F1 and the p53 homologue p73[29–33], or by the nutlin-3-induced inhibition of the drug

transporter P-glycoprotein.[16] In any case, these observations show that the anticancer activity of nutlin-3 does not strictly rely on the existence of wt-p53. It should nevertheless be noted that nutlin-3 treatment affected mt-p53 SK-ES-1 cells to a significantly lesser extent, in keeping with the relatively lower efficacy of nutlin-3 in mt-p53 and p53-deficient cells found in the previous studies.[15,16,28,29,31,33]

p53 suppresses tumour development and progression by initiating cell cycle arrest and, importantly, apoptosis, but it is not fully understood how the decision between these outcomes in response to p53 activation is made.[26] In a study employing ten randomly selected cancer cell lines, nutlin-3 was found to promote cell cycle arrest rather than apoptosis.[6] By contrast, our findings suggest that nutlin-3 treatment resulted in the initiation of apoptosis in wt-p53 ES cells. We observed that nutlin-3 triggered $\Delta\psi_m$ dissipation, caspase-3 activation and DNA fragmentation, three common features of apoptosis. In line with these results, nutlin-3 stimulated gene expression of PUMA, a crucial mediator of p53-induced apoptosis[34], in wt-p53 cells. Of note, however, our data show that SK-ES-1 cells were resistant to nutlin-3-mediated apoptosis, indicating that the observed antitumour activity of nutlin-3 in mt-p53 ES cells stemmed predominantly from the inhibition of cell growth.

As an accessory finding of the apoptosis determinations, nutlin-3-induced apoptosis emerged to proceed independently of caspase-8, for nutlin-3 evoked an apoptotic response in CADO-ES-1 cells, a cell line deficient in caspase-8 expression.[35] Caspase-8 was reported to be essential for p53-mediated apoptosis in ES cells.[36] However, this was inferred from the apoptosis-inhibitory effect of the putative caspase-8-specific inhibitor z-IETD-fmk, which is now known to unselectively block the activity of several caspases, including the effector caspases-3 and -7.[37,38]

In addition to eliciting apoptosis, we found nutlin-3 also capable of inducing cellular senescence in ES cells. The chemotherapy-mediated regression of cancers has long been attributed to the drugs' ability to initiate apoptosis, but it has recently been recognised that the induction of cellular senescence – an irreversible cell cycle arrest – can contribute to the therapeutic efficacy of antineoplastic agents.[39] In particular, restoration of p53 activity has been shown to produce tumour regression by inducing senescence rather than apoptosis in a mouse liver carcinoma model.[40] Our finding reveals that nutlin-3 activates pleitropic anticancer mechanisms in ES, suggesting that nutlin-3 may be effective in the treatment of tumours with curtailed apoptotic responsiveness.

Nutlin-3 has been demonstrated to synergise with other antineoplastic agents, e.g. various cytostatics[14,16,19,21,29,32,33], the apoptosis-inducing cytokine TRAIL[41], the proteasome inhibitor bortezomib[42] and histone deacetylase inhibitors.[43] However, the combination of nutlin-3 with inhibitors of NF-κB transcription factors has not yet been explored. The rationale of this approach is based on the fact that aberrant NF-κB activity is involved in cancer development and progression.[44] Accordingly, NF-κB inhibitors have been reported to be active against malignant cells as single agents and in combination with other anticancer regimens. In ES cells, inhibition of NF-κB was shown to enhance the apoptotic activity of TNF-α[45] and histone deacetylase inhibitors.[46] Here, we have shown that the NF-κB inhibitor CAPE augmented nutlin-3-induced cell killing, thus supporting the concept of simultaneous targeting of p53 and NF-κB as antitumour strategy.[27]

From a clinical perspective, nutlin-3's ability to activate p53 in a non-genotoxic manner, thus bypassing the DNA damage associated with cytotoxic anticancer agents, is of substantial interest. In this regard, nutlin-3 may be especially advantageous for the treatment of childhood malignancies. The introduction of intensive chemotherapy has improved the prognosis of childhood neoplasias, but the deleterious long-term side-effects of the current genotoxic treatment regimens are becoming serious problems for survivors of childhood cancer.[47] In particular, ES survivors are at a high risk for late therapy-related morbidities, predominantly second primary malignancies and cardiac dysfunction.[48] Therapeutic modalities with less genotoxic drugs, therefore, are highly desirable. Our results presented here establish the potential of nutlin-3 for the treatment of ES.

Conflict of interest statement

There exists no conflict of interest.

Note added in proof

While this paper was under review, a similar set of findings was published by Pishas et al.[49] Both the results and implications of this study are in good agreement with our data.

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