Parthenolide Complements the Cell Death-inducing Activity of Doxorubicin in Melanoma Cells

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Abstract. *Background: Melanoma is characterized by high resistance to chemotherapy. The aim of this study was to investigate combined effects of doxorubicin and parthenolide on melanoma cells. Materials and Methods: Thiazolyl blue tetrazolium bromide (MTT) assay and flow cytometry were used to evaluate viability. The p53 levels and Poly-ADP ribose polymerase (PARP) cleavage were assessed by western blot. Electrophoretic mobility shift assay (EMSA) and quantitative real-time polymerase chain reaction (qRT-PCR) were used to evaluate changes in nuclear factor-κB (NF-κB) activity and gene expression, respectively. Results: Both drugs reduced the viability of melanoma cells and induced apoptosis. Expression of the ATP-binding cassette sub-family B member-5 (ABCB5) transporter was enhanced by doxorubicin. Doxorubicin induced activity of p53 and NF-κB. Parthenolide markedly reduced the constitutive and doxorubicin-induced NF-κB activity measured as the nuclear NF-κB, and expression of matrix metalloproteinase-9 (MMP9) and it had no effect on p53. Discussion: Doxorubicin and parthenolide affected distinct pathways in melanoma, and parthenolide was capable of combating some pro-survival effects of doxorubicin in the combined treatment. This provides a rationale for in vivo investigation of this drug combination.*

Aberrant activity of transcription factors plays crucial roles both in tumour development and tumour response to treatment. The efficacy of many drugs is reduced because they stimulate the anti-apoptotic activity of nuclear factor-κB (NF-κB) (1). Hyperactivated NF-κB was shown to repress the stability of p53 by direct up-regulation of murine double minute-2 (MDM2) (2) and by competing for limited pools of p300 and CREB-binding protein (CBP) (3). This impairs p53 function in cell -cycle arrest, senescence and apoptosis. A drug, or drug combination, that can simultaneously inhibit NF-κB and activate p53 would be of great interest. Some drugs have demonstrated this dual activity. For example, quinacrine and other derivatives of 9-aminoacridine were found to be capable of simultaneously activating p53 and repressing NF-κB in renal cell carcinoma (4). Nutlins were shown to have the potential to stabilize p53 by inhibiting MDM2 and to suppress TNF-α-induced activation of the NF-κB reporter genes in lung cancer cells (5).

Parthenolide has pleiotropic effects against several cancer cell lines and xenografts (reviewed in 6-8). It has been shown to affect transcription factors participating in deregulated pathways of cell survival, proliferation and apoptosis. Parthenolide diminished the activity of NF-κB (9-14), influenced signal transducer and activator of transcription-3 (STAT3) activity (15), induced sustained c-Jun N-terminal kinase (JNK) activity (16, 17) and enhanced nuclear translocation of hypoxia-induced transcription factor -1α (HIF-1α) (18).

Our group and others have shown that parthenolide efficiently induces cell death in melanoma cell lines (19-21). Our previous study revealed that parthenolide reduced the constitutive and cisplatin-induced NF-κB activity, and down-regulated NF-κB-dependent gene expression. This combined treatment with cisplatin, however, did not induce cell death more efficiently than parthenolide alone. Recently, we showed that parthenolide markedly reduces the frequency of cells expressing the ATP-binding cassette sub-family B member-5 (ABCB5) transporter and eradicates melanoma cells with self-renewing capacity (22). In the current study, we used partenolide in combination with doxorubicin. Doxorubicin induces genotoxic stress through a different pathway from cisplatin, however, it also activates NF-κB (23). We were interested to determine whether parthenolide could diminish the doxorubicin-triggered NF-κB activity, and induce p53 in melanoma cells. We employed the melanoma cell lines, A375 and 1205Lu, and cells derived from surgical specimens of nodular melanoma.

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Materials and Methods

Drugs. Doxorubicin was obtained from Sigma-Aldrich (St. Louis, MO, USA), and parthenolide from BIOMOL International (Exeter, UK). Parthenolide was dissolved in dimethyl sulfoxide (DMSO). Doxorubicin was dissolved in sterile distilled water. Thiazolyl blue tetrazolium bromide (MTT) was purchased from Sigma-Aldrich.

Cells and culture conditions. A375 and 1205Lu cells (a gift from Prof. Laidler, Jagiellonian University, Cracow, Poland) were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. DMBC8 and DMBC10 cell lines derived from nodular melanoma specimens were maintained in culture as described previously (24).

Cell viability. The MTT assay was used to assess the viable cell number in cultures of melanoma cells treated with 10 μM parthenolide and 1 or 5 μM doxorubicin used alone or in combinations. Cells were seeded in 96-well plates, and 6 h later cells were exposed to the tested compounds for 16 h, 22 h and 44 h. MTT reagent (0.84 mg/ml) was then added to each well and cells were incubated for 3 h. The absorbance was determined at 540 nm using a microplate reader (Infinite M200 PRO; TECAN, Grodig, Austria).

Annexin V staining. Annexin V staining was employed to assess the percentages of apoptotic cells in melanoma cultures after 22 h of drug treatment. Cells were washed and then incubated for 15 min with 100 μl staining solution containing 2 μl of fluorescein isothiocyanate (FITC)-conjugated annexin V (Roche Diagnostics, Mannheim, Germany). Cells were analyzed by flow cytometry using a FACSVerse flow cytometer (Becton Dickinson, San Jose, CA, USA). The results were processed using FACS Suite software (Becton Dickinson).

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA). Melanoma cells were treated with either 0.08% DMSO (control) or 24 μM parthenolide for 3 h, or doxorubicin at 15 μM or 30 μM for 2 h. For drug combinations, melanoma cells were pre-treated with parthenolide at 24 μM for 1 h, and then doxorubicin was added at 15 μM or 30 μM for additional 2 h. Nuclear extracts were prepared as described previously (25). A double-stranded oligonucleotide 5' AAT TAG TTG AGG GGA CTT TCC CAG GC 3' containing the NF-κB-binding motif was end-labelled by (α-^{32}P)-dATP using Sequenase 2.0 (USB Corporation, Cleveland, OH, USA) and purified as described previously (25). After electrophoresis on native 5% PAG in 0.5× TBE Buffer, bands were visualized by phosphoimaging on Molecular Imager® FX (Bio-Rad, Hercules, CA, USA). The specificity of complexes was confirmed by using a 100-fold excess of cold NF-κB oligonucleotide as a specific competitor, or non-labeled oligonucleotide with an unrelated sequence: 5'-AAT TGA ACA TGT CTA AGC ATG CTG-3'. For the supershift experiments, nuclear extracts were pre-incubated at room temperature for 5 min with 4 μg anti-p50 (sc-7178 X), anti-p65 (sc-372 X) and IgG (sc-2027) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Preparation of cell lysates and western blotting. Melanoma cells were lysed in RIPA buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] containing protease and phosphatase inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μM leupeptin, 0.5 mM Na_3VO_4, and 10 mM NaF] for 30 min at 4˚C. Protein concentration was determined by the Bradford assay (BioRad). Protein (15 μg) was loaded on 8% SDS-polyacrylamide gels and the electrophoresis was carried out at constant voltage of 25 V/cm. The proteins were transferred onto Immobilon-P PVDF Transfer Membrane (Merck Millipore, Billerica, MA, USA) at constant voltage (100 V, 1 h, 4˚C) using BioRad transfer system. The efficacy of transfer was confirmed by Ponceau S staining. The immunodetection was carried out using primary antibodies against p53, poly-ADP ribose polymerase (PARP) (Santa Cruz Biotechnology), p-p53 (Cell Signaling, Beverly, MA, USA), and β-actin as a loading control (Sigma-Aldrich), followed by binding of the secondary horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit antibodies (sc-2005 and sc 2004, respectively; Santa Cruz Biotechnology). The proteins were visualized on a medical X-ray film (Foton-Bis, Bydgoszcz, Poland) using Pierce® ECL Western Blotting Substrate (Pierce, Rockford, IL, USA). The results were analyzed densitometrically using Quantity One Software (BioRad).

Total RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR). The quantification of mRNA expression of appropriate genes was performed by qRT-PCR, as described in detail by Szulawska *et al*. (27). Total RNA was collected using a total RNA isolation kit (A&A Biotechnology, Gdynia, Poland). The RNA concentration and purity was evaluated by determining absorbance with a Tecan NanoQuant Plate reader (Tecan Group Ltd., Männedorf, Switzerland). Total RNA (1 μg) was reverse transcribed into cDNA using random hexamers and SuperScript II Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA). Amplification reactions were performed using DyNAmo Flash SYBR Green qPCR Kit (Finnzymes, Vantaa, Finland), 350 nM primers and 25 ng DNA template per reaction. The primers used for real-time PCR were as following: *ABCB5*: 5'-GAC CTC CTG CTG CCT ATG AG-3' and 5'-ATG TGC CCA TTA AGC CTT TG-3'; matrix metalloproteinase-9 (*MMP9*): 5'-GAC CAG GAC AAG CTC TAC GG-3' and 5'-CAG AAG CCC CAC TTC TTG TC-3'. The annealing temperature for all genes was 55˚C. Gene expression levels were tested using the Rotor-Gene 3000 Real-Time DNA analysis system (Corbett Research, Morklake, Australia). The relative expression was based on the expression ratio of the target genes versus the reference gene ribosomal protein S17 (*RPS17*; QuantiTect Primer Assay; Qiagen, Valencia, CA, USA).

Statistical analysis. Obtained data represent the means±S.D. from at least three independent experiments. The significance was validated using Student's unpaired *t*-test. The difference was considered significant when the *p*-value was less than 0.05.

Results

Parthenolide and doxorubicin reduced viability of melanoma cells. To evaluate the response of melanoma cells to 10 μM parthenolide and 1 or 5 μM doxorubicin, used alone and in combination, we employed the melanoma cell lines: A375, 1205Lu, DMBC8 and DMBC10. As shown in Figure 1A, parthenolide and doxorubicin used as single agents for 16 h significantly ($p<0.05$) reduced the viability of melanoma

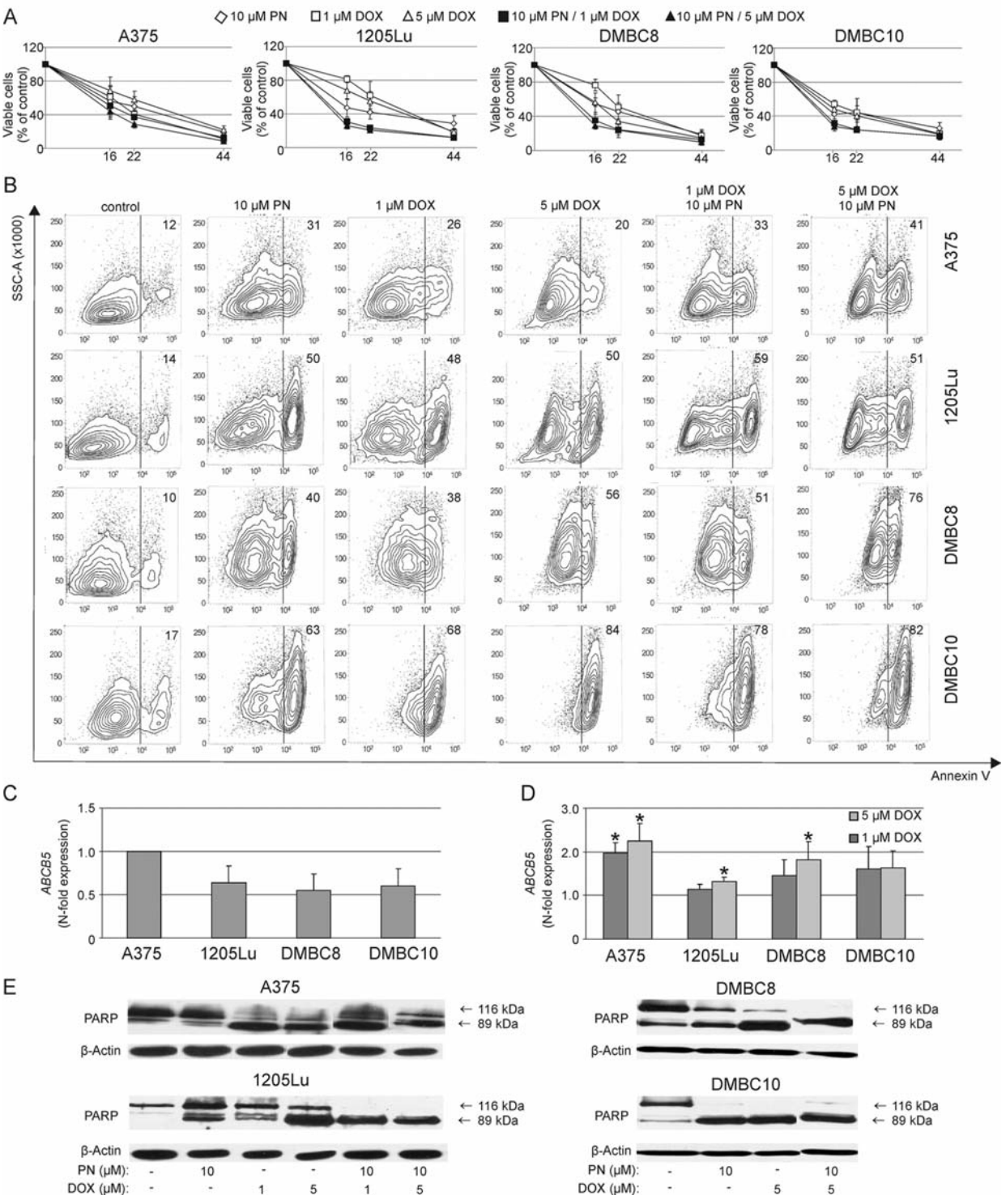

Figure 1. *Parthenolide (PN) and doxorubicin (DOX) induced cell death in melanoma cultures. A: Parthenolide and doxorubicin decreased viability of melanoma cells as assessed by the thiazolyl blue tetrazolium bromide (MTT) assay. Data represent the means±S.D. ($p<0.05$; n=3). B: Flow cytometric analysis showing that parthenolide and doxorubicin induced apoptosis of melanoma cells. The numbers indicate the percentage of annexin V-positive cells in a typical experiment (n=3). C: The basal ATP-binding cassette sub-family B member-5 (ABCB5) expression in 1205Lu, DMBC8 and DMBC10 cells in comparison with the basal expression in A375 cells, measured by quantitative real-time polymerase chain reaction (qRT-PCR). Data represent the means±S.D. ($p<0.05$; n=3). D: Doxorubicin enhanced the expression of ABCB5 as analyzed by qRT-PCR. Data represent the means±S.D. (*$p<0.05$, significantly different from untreated control; n=3). E: Increased poly-ADP ribose polymerase (PARP) cleavage was used as a molecular indicator of apoptosis. Representative immunoblots are shown (n=2).*

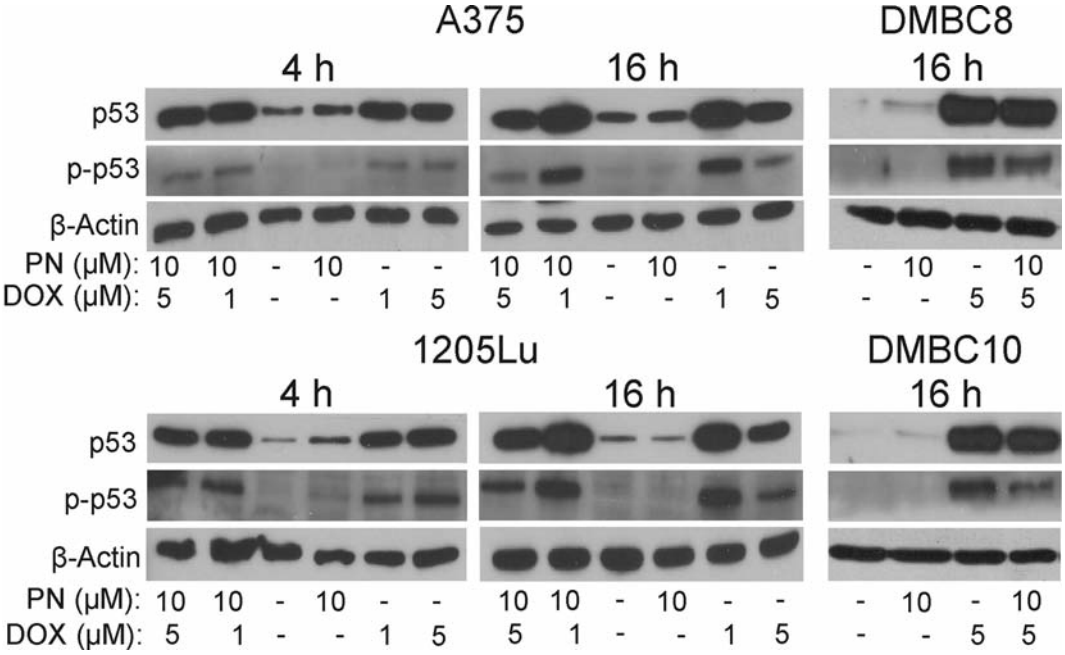

Figure 2. *Doxorubicin (DOX), but not parthenolide (PN), activated p53 in melanoma cells. Western blotting shows the total level of p53 and its activated Ser15-phosphorylated form. Representative immunoblots are shown (n=2).*

cells, which was further reduced by combined treatment.

We have previously shown that parthenolide induces the intrinsic apoptotic pathway in melanoma cells (19). In that report, parthenolide substantially increased the percentage of annexin V-positive cells, and induced mitochondrial membrane potential dissipation and caspase-3 activity. In the current study, doxorubicin markedly increased the percentage of annexin V-positive cells (Figure 1B). Doxorubicin, both alone and in combination with parthenolide, was less effective in A375 than in 1205Lu, DMBC8 and DMBC10 cells. The high basal expression of ABCB5 drug transporter in A375 cells relative to that in the other cell lines (Figure 1C), which was in addition significantly augmented by doxorubicin (Figure 1D), may cause a more efficient removal of doxorubicin, consequently reducing its intracellular concentration and efficacy. Western blots for full-length PARP (116 kDa) and its cleaved form (89 kDa) revealed that parthenolide and doxorubicin induced PARP cleavage (Figure 1E), confirming parthenolide- and doxorubicin-induced apoptosis.

Doxorubicin, but not parthenolide, strongly activated p53 in melanoma cells. Parthenolide failed to induce expression of total and active Ser-15-phosphorylated-p53 (Figure 2). Doxorubicin induced p53 expression and phosphorylation of p53 in all melanoma cell populations as soon as 4 h from treatment. Densitometric analysis revealed that 10 μM parthenolide changed the p53 level from 0.9-fold in 1205Lu cells to 1.4-fold in DMBC8 and DMBC10 populations, whereas 5 μM doxorubicin caused a significant increase of p53, from 3.1-fold in A375 cells to 12.3-fold in DMBC8 cells. There were no significant differences between p53 levels in cells treated with doxorubicin alone and in combination with parthenolide. This suggests that parthenolide did not induce p53 but also did not block p53 induction.

Parthenolide inhibited constitutive and doxorubicin-induced NF-κB activity in melanoma cells. NF-κB acted as a p50-p65 heterodimer in melanoma cells, as both subunits were recognized by appropriate antibodies (Figure 3A). Doxorubicin enhanced the NF-κB binding. We investigated whether pre-treatment with parthenolide could prevent doxorubicin-mediated activation of NF-κB in melanoma cells. Melanoma cells were either i) incubated with parthenolide for 3 h, ii) incubated with doxorubicin for 2 h or iii) pre-treated with parthenolide for 1 h followed by doxorubicin incubation for additional 2 h. Parthenolide efficiently reduced both constitutive and doxorubicin-induced NF-κB activity (Figure 3A). The influence of both drugs on NF-κB activity was also assessed by qRT-PCR by measuring changes in the expression of *MMP9*, an NF-κB-dependent gene. Doxorubicin significantly ($p<0.05$) increased the level of *MMP9* transcript (Figure 3B), and parthenolide significantly reduced doxorubicin-enhanced expression of *MMP9*. Altogether, these

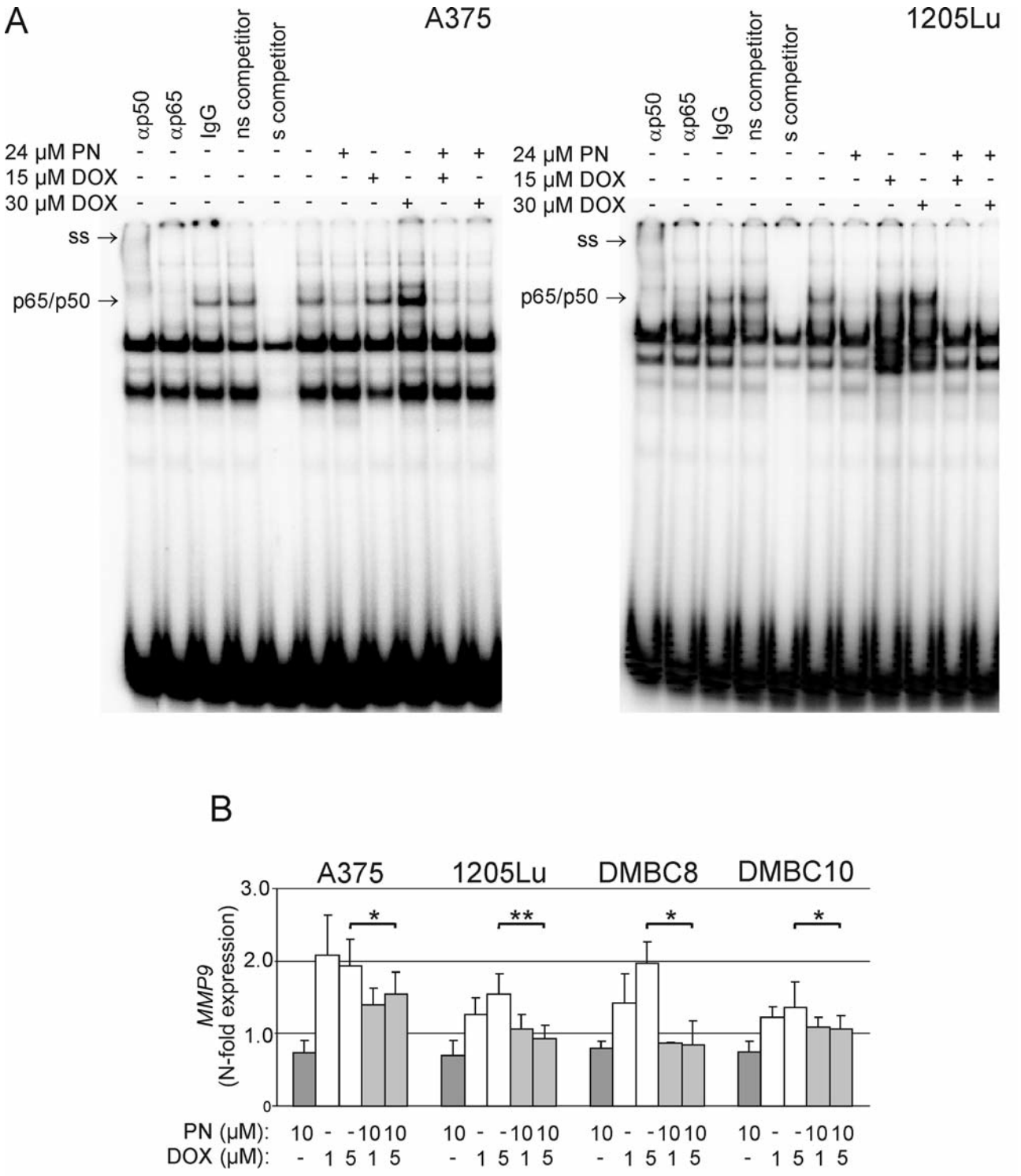

Figure 3. *Parthenolide (PN) inhibited constitutive and doxorubicin (DOX)-induced nuclear factor κB (NF-κB) activity in melanoma cells. A: The nuclear level of NF-κB was evaluated in melanoma cells by electrophoretic mobility shift assay (EMSA). Melanoma cells were incubated with parthenolide or doxorubicin alone at the indicated concentrations, or were pre-incubated with parthenolide and then treated with doxorubicin. The arrows indicate the NF-κB–DNA-specific complex (p50/p65 dimer) and the complex supershifted by appropriate antibodies (ss). ns, Non-specific; s, specific oligonucleotide. B: Changes in the expression of matrix metalloproteinase-9 (MMP9), a NF-κB-dependent gene, were analysed with qRT-PCR. Data represent the means±S.D. (*p<0.05, **p<0.01; n=3).*

results indicated that parthenolide can effectively reduce constitutive and doxorubicin-induced NF-κB activity in melanoma cells, measured as the level of NF-κB in the nucleus and the expression of *MMP9*.

Discussion

Combined treatment has been recognized as being necessary against tumours that benefit from many activated anti-apoptotic pathways. The constitutively active IκB kinase (IKK)/NF-κB pathway is an important pro-survival signaling pathway in melanoma cells (28). In addition, even if the majority of melanomas retain wild-type p53, the signaling pathways downstream of p53 leading to cell-cycle arrest and apoptosis seem to be defective (29). Many drugs simultaneously activate p53 and NF-κB, and as both transcription factors counteract each other's function, drug efficacy in triggering cell death is diminished (30). Results obtained for nutlin-3 (which suppressed the NF-κB pathway only in cells with wild-type p53), *R*-roscovitine [CYC202; which inhibited NF-κB independently of the p53 status of the cancer cells (31, 32)], and pifithrin-α [which inhibited p53 and enhanced the expression of NF-κB (33)] highlight the importance of the interplay between these two transcription factors in the cellular response to drug treatment. Parthenolide reduced the activity of NF-κB in many types of cancer cells (11, 12, 19) and induced p53 activity (13, 14). The inhibition of NF-κB with parthenolide occurred *via* targeting the IKK complex (9), whereas the activation of p53 was due to abrogation of MDM2-p53 complex formation (14). Parthenolide promoted p53 release and nuclear accumulation, followed by acetylation of Lys-382 by p300 histone acetyltransferase and activation through Ser-15 phosphorylation (34). An increase in the level of p53 after parthenolide treatment was observed only in some cell lines of hepatocellular carcinoma (13), suggesting that a more detailed analysis would be necessary to find the prerequisites for parthenolide-driven p53 activation in cancer cells. In our study, parthenolide suppressed constitutive and doxorubicin-driven NF-κB activation, but did not significantly change the level and activity of p53 measured by phosphorylation of Ser-15. Thus, together with previously published data, the current results indicate that parthenolide-dependent inhibition of NF-κB activity is universal and can occur in many types of cancers, whereas the influence of parthenolide on p53 should be recognized as being cancer-type specific.

Doxorubicin-alone was not highly effective against melanoma cells *in vitro* (35) and also failed as monotherapy in clinical trials in patients with metastatic malignant melanoma (36, 37). In melanoma cells, the high expression of ABCB5 transporter, which acts as a drug-efflux pump for many cytotoxic compounds, including doxorubicin (38), might be responsible for the reduction of cytotoxic stress. In the current study, doxorubicin even enhanced the expression of the ABCB5 transporter.

Based on our current and previous findings, we propose that doxorubicin would be more toxic to melanoma cells when its undesired effects such as NF-κB activation and stimulation of ABCB5 expression, are counteracted by parthenolide. It has been already demonstrated that kinase inhibitor of NF-κB-1 (KINK-1), another inhibitor of the IKK/NF-κB module, increases the apoptotic sensitivity of melanoma cells to doxorubicin (23). In mouse models of melanoma chemotherapy, the ablation of melanoma-intrinsic NF-κB activity with BMS-345541, a small-molecule inhibitor of IKK, resulted in apoptosis-driven tumour regression after treatment with doxorubicin (39). Most recently, it has been demonstrated that doxorubicin-induced activation of NF-κB in melanoma cells can be abrogated specifically by inhibition of IKKβ but not IKKα (40). Parthenolide, as a potent inhibitor of both IKKβ and IKKα (10), might be considered a more universal inhibitor of NF-κB activity. Moreover, we recently showed that parthenolide reduced the frequency of ABCB5-positive cells (22) and enhanced dacarbazine activity against melanoma cells (41). It is reasonable to assume that counteracting some undesired effects of doxorubicin with parthenolide may increase the anticancer efficacy of doxorubicin *in vivo*. Thus, it remains to be elucidated whether parthenolide used in combination with doxorubicin might be effective in melanoma *in vivo*.

Conflicts of Interest

None declared.

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