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Curcumin and Resveratrol Induce Apoptosis and Nuclear Translocation and Activation of p53 in Human Neuroblastoma

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Abstract. *Background: Neuroblastoma (NB) is an aggressive childhood cancer of the peripheral nervous system arising from neural crest sympathoadrenal progenitor cells. Despite current rigorous treatment protocols, prognosis for high stage NB patients is poor and so there remains a need for more effective, less cytotoxic treatments. Curcumin and resveratrol possess anti-tumor properties in adult cancer models and negligible toxicity in normal cells, but little is known about the effect of these agents on pediatric cancers. Materials and Methods: Stage 4 MYCN-amplified NB cell lines, with wild-type or mutant p53, were treated with curcumin and resveratrol and analyzed for effects on proliferation, cell cycle, induction of apoptosis and p53 function. Results: Treatment induced a dose- and time-dependent decrease in cell viability, cell cycle arrest and induction of apoptosis. Treatment transiently up-regulated p53 expression and induced nuclear translocation of p53, followed by induction of $p21^{WAF\text{-}1/CIP\text{-}1}$ and Bax expression. Conclusion: Observations suggest that the cytotoxicity, cell cycle arrest and apoptosis induced by curcumin and resveratrol in NB cells may be mediated via functionally activated p53 and merit further study.*

Neuroblastoma (NB) is a cancer of the sympathetic nervous system and the third most common childhood malignancy, accounting for 15% of cancer-related mortality in children (1). Stage 4 NB diagnosed without *MYCN* amplification before the age of 1 has an excellent prognosis. In contrast, treatment of patients with high-risk, *MYCN*-amplified, stage 4 NB diagnosed after 1 year of age is successful in less than half the patients, with a five-year overall survival of about 25% and an event-free survival of less than 20% (2), despite improved survival by the utilization of intensified and higher doses of a variety of chemotherapeutics, autologous bone marrow transplantation and adjuncts like retinoids (3). The etiology of NB is postulated to stem from the arrested differentiation of its neural crest progenitor cells (4). These highly malignant tumors exhibit a number of frequent genetic alterations including *MYCN* amplification or overexpression, 1p36 LOH, 17q gains and other alterations at lower frequency which appear to drive the malignant phenotype (5, 6). *TP53*, the most commonly mutated gene in human cancer and altered in a wide variety of sporadic tumors (7), is rarely mutated in NB (8, 9).

Unlike most tumors, the p53 protein is wild-type in the overwhelming majority of NB cases (9) and is primarily cytosolic (10), where it is abnormally stabilized and overexpressed (11), in the absence of stress. The accumulation and subcellular compartmentalization of p53, its physical stabilization and exclusion from the nucleus has been proposed as a mechanism for the functional inactivation of p53 in neuroblastoma (11-13), although a number of studies reveal that the p53 signaling pathway is potentially functional in various neuroblastoma cell lines (13-16). In contrast to undifferentiated NB (17), differentiation of NB cells is associated with down-regulation of overexpressed p53 to normal cellular levels (10, 11, 17), a concomitant conversion of the sequestered phenotype to the wild-type phenotype and, importantly, a dramatic rise in the ability to undergo apoptosis (18, 19). This differentiation-associated conversion suggests a reversible process and a possible reconstitution to functional p53.

p53 functions as a negative regulator of cellular proliferation at the G1/S-phase of the cell cycle (20). p53 acts through the transactivation and repression of numerous downstream genes including crucial growth inhibitory genes such as the Cdk inhibitor $p21^{WAF\text{-}1/CIP\text{-}1}$, Bax and IGF-BP3, or *via* p53 activity not requiring transcription (protein-protein interactions) (21). p53 is activated in response to various stress signals and several responses may be provoked by p53, including cell cycle arrest, senescence, differentiation and apoptosis, with the option chosen being

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Key Words: Neuroblastoma, p53, curcumin, resveratrol, apoptosis.

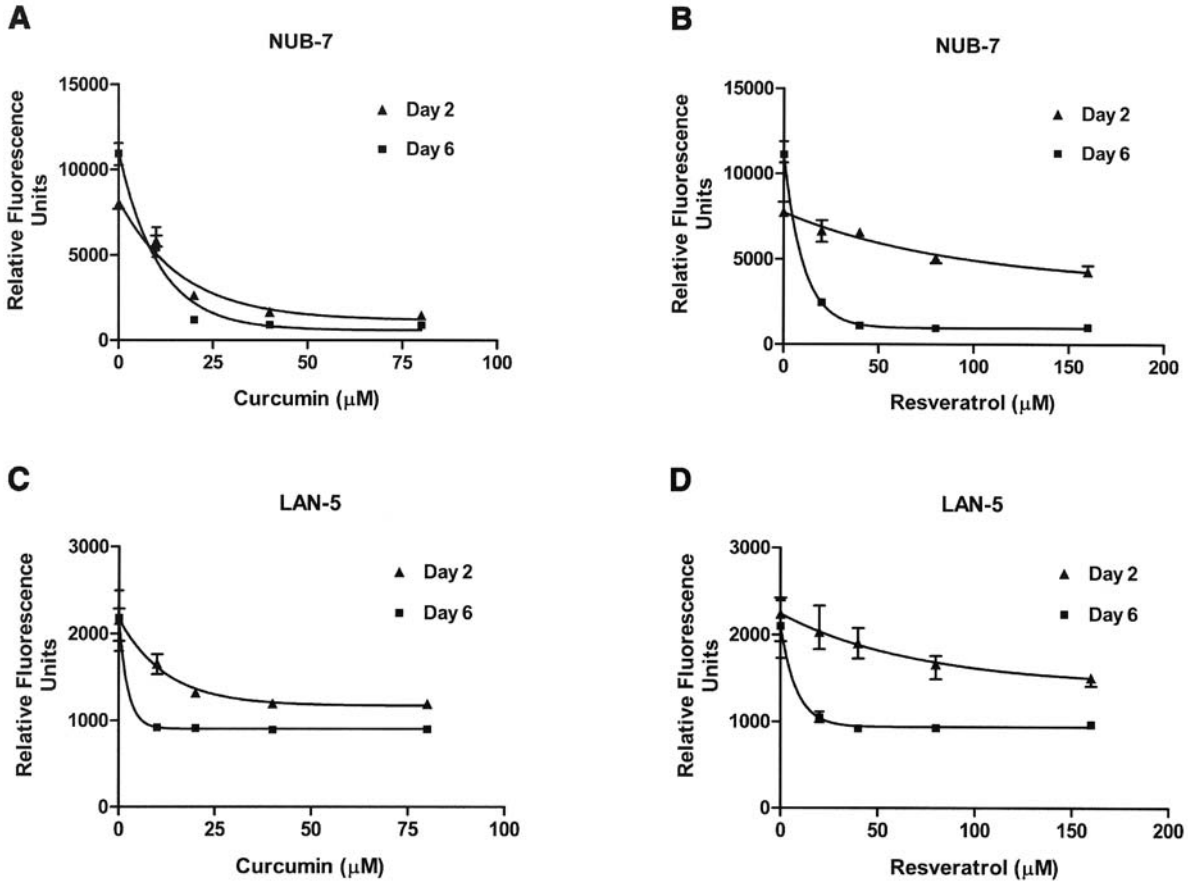

Figure 1. *Effects of curcumin and resveratrol on the growth of NUB-7 (A and B) and LAN-5 (C and D) cells. Cells were treated with various concentrations of curcumin and resveratrol for 2-6 days and cytotoxicity was measured using AlamarBlue. Increasing cytotoxicity, measured as a reduction in relative fluorescence units, was observed with increased time and dose of treatment. Values are the means of three experiments, each assayed in triplicate. Bars, SD.*

dependent on many intrinsic and extrinsic cellular factors. In most cases, induction and activation of p53 leads to an irreversible inhibition of cell growth and activation of apoptosis (21). Since p53 mediates cell killing in chemotherapy and radiotherapy (22), the possibility of chemically restoring the functional activity of p53 is of obvious significance in cancer therapy.

Plant-derived polyphenolic compounds including curcumin and the stilbene, resveratrol, possess a wide range of pharmacological properties, the mechanisms of which have been the subject of considerable interest. They are recognized as naturally occurring antioxidants and anti-inflammatories and have been implicated as anti-tumor compounds (23). These dietary polyphenolics induce apoptosis in various cancer cell lines but not in normal fibroblasts (24). Recent studies have demonstrated that p53-dependent apoptosis can be triggered upon treatment of various cancer cell lines with curcumin and resveratrol (25, 26). The fact that these polyphenolic compounds are ingested by human populations as part of the normal diet in relatively high concentrations without adverse reactions points to their high potential as putative chemopreventive or therapeutic agents.

We now show that curcumin and resveratrol exhibit a dose- and time-dependent cytotoxic effect on *MYCN*-amplified NB cell lines with induction of cell cycle blocks in G2/M after treatment with curcumin, and S-phase after treatment with resveratrol, and induction of a strong dose-dependent apoptotic response. Suppression of the cell cycle and induction of apoptosis by these compounds was accompanied by nuclear accumulation of p53 and the induction of the cyclin-dependent kinase inhibitor $p21^{WAF-1/CIP-1}$ and Bax, a pro-apoptotic factor. Our demonstration that curcumin and resveratrol have potent growth and survival inhibitory effects on NB and act *via* activation of p53 supports further study in NB.

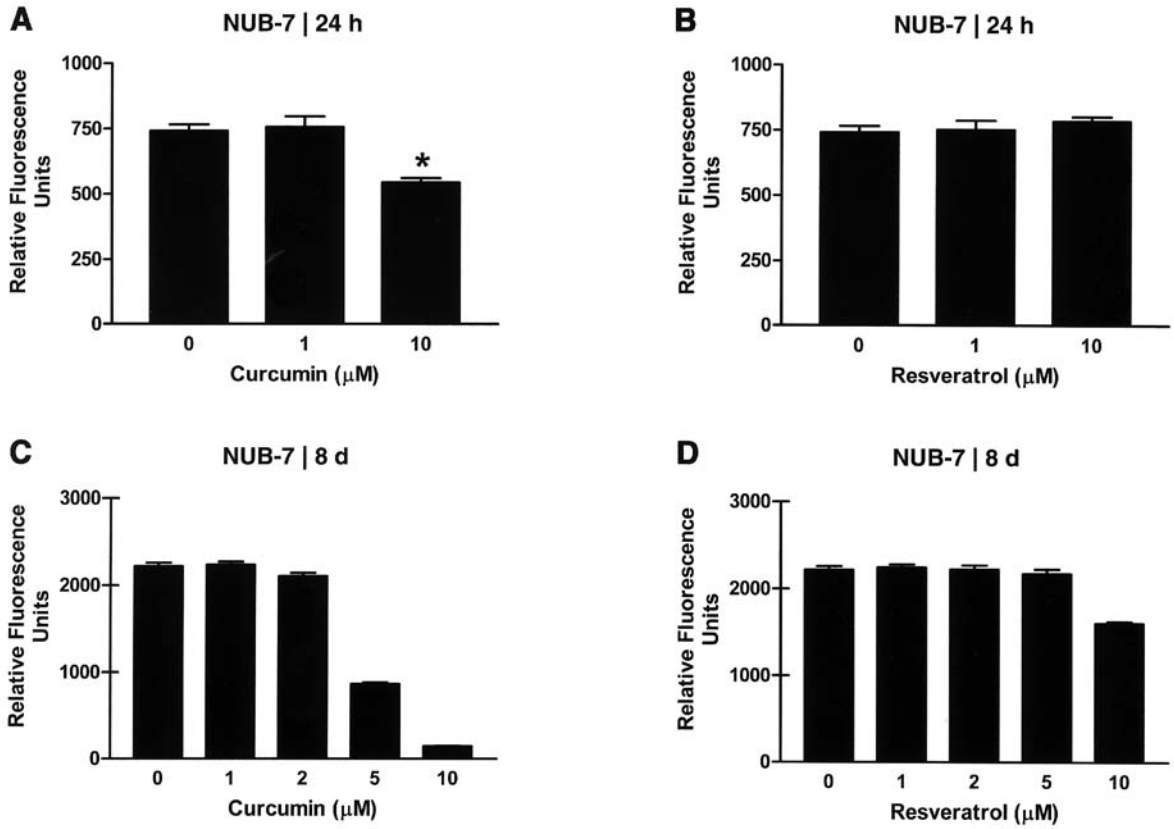

Figure 2. *Effects of low-dose curcumin and resveratrol treatment on NUB-7 cells. In A and B, treatments were repeated every 6 h for a 24-h period. Increased cytotoxicity, quantified by AlamarBlue Assay, was observed with 10 μM curcumin. In C and D, treatments were repeated every 2 days over a period of 8 d. Increased cytotoxicity was observed predominantly with curcumin. Each condition was tested in triplicate in two independent experiments. * indicates significant difference relative to control, $p<0.05$. Bars, SD.*

Materials and Methods

Cell lines. The selection of stage 4 NB cell lines for this study was based on *MYCN* and *TP53* status. NUB-7 (27), LAN-5 (obtained from Drs. R.C. Seeger and P. Reynolds) and IMR-32 are *MYCN*-amplified (~25-50 copies) with wild-type p53. SK-N-BE(2) is similarly *MYCN*-amplified but carries a *TP53* mutation at codon 135 with a nucleotide change of TGC → TTC, corresponding to an amino acid substitution of Cys by Phe (3). NUB-7, LAN-5, IMR-32, SK-N-BE(2) and the reference cell lines, p53-null osteosarcoma SAOS-2 and wild-type p53 fibrosarcoma HT1080 (ATCC), were maintained in alpha-MEM supplemented with 20% fetal bovine serum and 1% penicillin/streptomycin, and incubated in a humidified chamber at 37˚C and 5% CO_2. The reference mutant p53-positive Wilms tumor cell line WiT-49 (28) was maintained in Iscove's DMEM supplemented with 15% calf serum (Hyclone, Logan, UT, USA), 2 mM L-glutamine, penicillin/streptomycin (100 μl/ml and 100 μg/ml, respectively) and 4 μl/L β-mercaptoethanol and 1.1 μg insulin/ml). Since the use of penicillin/streptomycin in culture can lead to masking of mycoplasma contamination, cultures were routinely tested for contamination by DAPI (Roche, Mississauga, Canada) fluorescence labeling.

Reagents and antibodies. Curcumin (Sigma, St. Louis, MO, USA) and resveratrol (Cayman Chemical, Ann Arbor, MI, USA) were prepared as 100 mM stock in DMSO and diluted as required in warmed fresh medium prior to application. The general protein kinase inhibitor 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine, H7 (Sigma), was prepared as 25 mM stock in water. The mouse monoclonal p53 antibody PAb-8 (cocktail of clones DO-7 and BP53-12) was obtained from NeoMarkers (Fremont, CA, USA); mouse monoclonal β-actin antibody was obtained from Sigma; mouse monoclonal $p21^{WAF1/CIP1}$ (F-5) antibody from Santa Cruz (Santa Cruz, CA, USA); rabbit polyclonal Bax antibody from PharMingen (Franklin Lakes, NJ, USA).

Cytotoxicity assay. Cytotoxicity was determined with AlamarBlue (Biosource, Camarillo, CA, USA). AlamarBlue is a non-toxic redox indicator designed to quantitatively measure proliferative activity (29, 30). AlamarBlue acts as an intermediate in the final redox reaction in the respiratory chain (the final reduction of O_2 and cytochrome oxidase). Specifically, it is an oxidation-reduction indicator that both changes color (blue to pink) and fluoresces in response to reduction. Increasing cytotoxicity is measured as a reduction in relative fluorescence units (RFU) since decreased

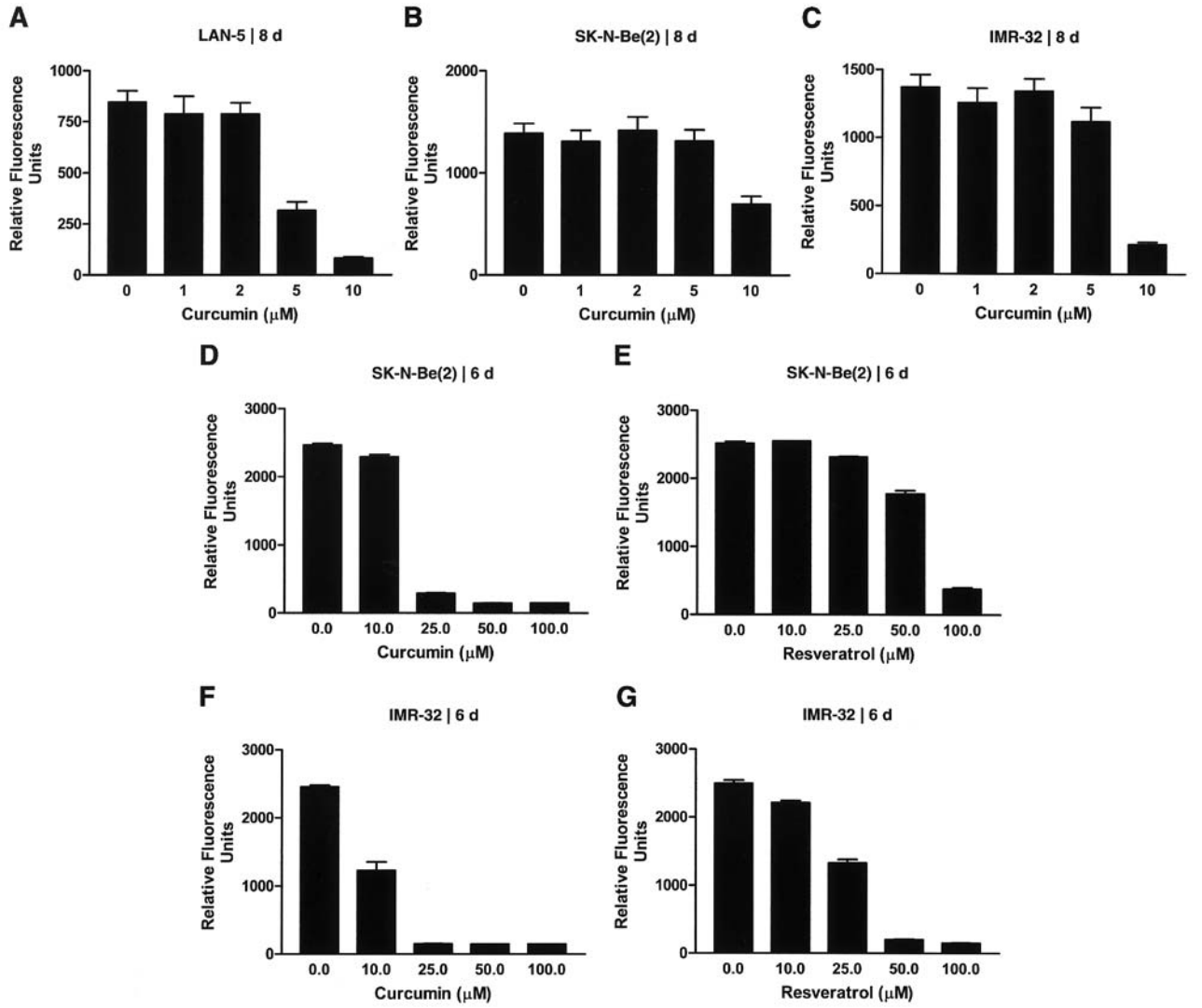

Figure 3. *Effects of curcumin and resveratrol treatment on NB cell lines LAN-5, SK-N-BE(2) and IMR-32. In A-C, low-dose curcumin treatment was administered over 8 days on LAN-5 (A), SK-N-BE(2) (B) and IMR-32 (C) cells. Increased cytotoxicity, measured by AlamarBlue Assay, was observed with 5 and 10 μM curcumin. Higher doses of curcumin and resveratrol applied over 6 days to SK-N-BE(2) (D and E) and IMR-32 (F and G) cells induced dose-dependent cytotoxicity. Note that SK-N-BE(2) was the least sensitive of the NB cell lines to curcumin and resveratrol. For each condition, the data were averaged from two separate experiments, each performed in triplicate. Bars, SD.*

mitochondrial activity results in decreased AlamarBlue reduction. Preliminary experiments in NB cells (not shown) confirmed that the relative fluorescence of AlamarBlue correlated with cell number (29, 30). Cells were plated at an initial seeding density of $2x10^5$ cells per well in 6-well plates and grown for 2 days. Cells were then treated with 0-80 μM curcumin or 0-160 μM resveratrol for 2-8 days. Following treatment, cells were washed with fresh media and AlamarBlue indicator was added to each well at an amount equal to 10% of the media volume (1:9 indicator:media). Cells were incubated for an additional 3 h at 37˚C and fluorescence readings were obtained using a Gemini microplate reader (Molecular Devices, Sunnyvale, CA, USA) (emission 540 nm, excitation 590 nm, cut-off 570 nm). Experiments were conducted in triplicate three times and results analyzed and graphed using Prism GraphPad (GraphPad Software, San Diego, CA, USA). Data are presented as relative fluorescence units (RFU) *versus* treatment concentration.

FACS analysis. Cells were grown and treated with an initial seeding density of $5x10^5$ cells per T-75. Cells were trypsinized and washed 3 times with PBS. Nuclei were stained with propidium iodide for cell cycle analysis by DNA content, as described (31). The cells were resuspended in 50 μl growth media (alpha-MEM). To each sample, 0.9 ml of the staining solution (25 μg/ml propidium iodide in 0.05% sodium citrate) was added, followed by the slow addition of 0.1 ml 20% Triton X-100 in distilled water without pipeting.

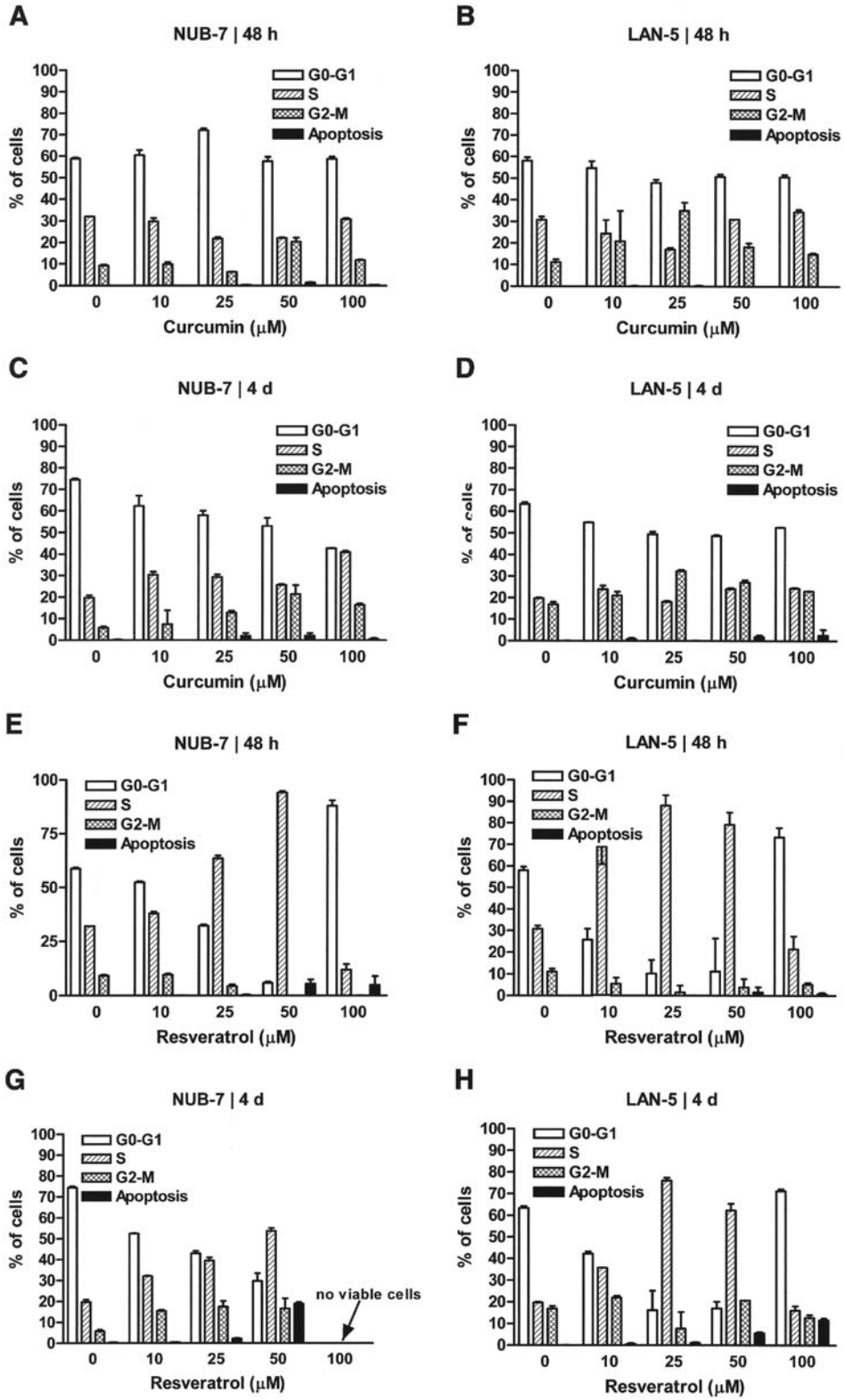

Figure 4. *Curcumin and resveratrol inhibited cell cycle progression in NUB-7 and LAN-5. Cells were treated over 48 h or 4 days, propidium iodide-stained DNA content was quantified by flow cytometry. The results for each condition are displayed as percentage of cells in each phase of the cell cycle. Essentially, curcumin (A-D) produced a block mainly in G2/M and resveratrol (E-H) induced a strong block in the S-phase. Results are representative of three independent experiments. Bars, SD.*

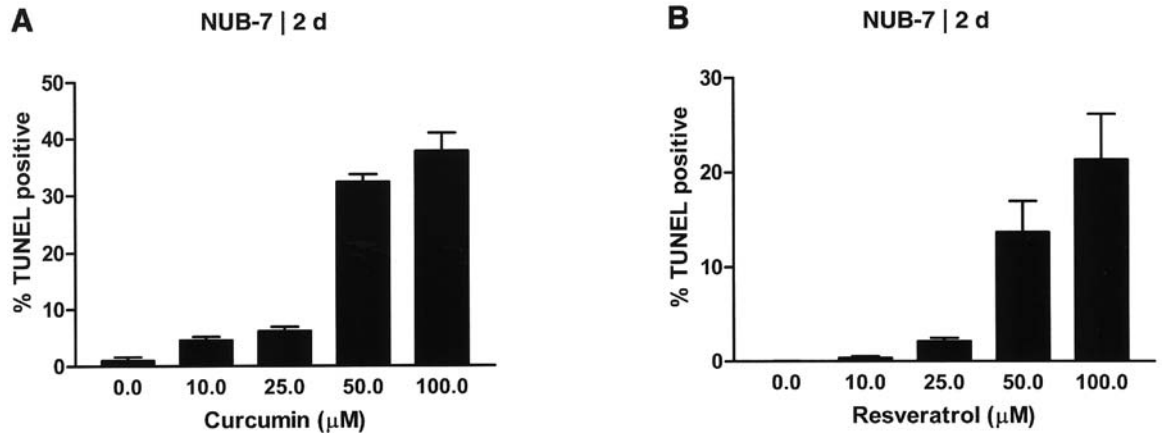

Figure 5. *Curcumin (A) and resveratrol (B) induced dose-dependent apoptosis in NUB-7. NUB-7 cells were treated for 48 h with 0-100 μM curcumin and resveratrol and harvested for TUNEL assay by flow cytometry as described in "Materials and Methods." TUNEL-positive, fluorescein dATP-labeled, cells were expressed as a percentage of total cells sampled. Bars, SD.*

After 30 min of gentle mixing by rotation, the tubes were placed at 4˚C in the dark overnight before analysis. Just before analysis, samples were filtered through a 100-mesh nylon textile and transferred to 12 x 75-mm polystyrene tubes. Isolated nuclei were then analyzed using a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). Data were collected in triplicate and analyzed and graphed using Prism GraphPad as percentage of cells in each phase of the cell cycle *versus* dose of treatment.

TUNEL assay. Measurement of DNA fragmentation was carried out by the terminal-deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end labeling assay (TUNEL, In Situ Cell Death Detection Kit, Roche, Mississauga, Canada), as described (32). Three million cells were treated in T-75 flasks for 2 days, harvested and passed through a 25-gauge needle, fixed in 4% paraformaldehyde for 1 h at room temperature and washed with cold PBS. Cells were permeabilized for 2 min at 4˚C with 0.1% Triton X-100 in sodium acetate and washed in cold PBS. PBS was removed and TUNEL reagent was added according to the manufacturer's instructions and incubated for 1 h at 37˚C. Cells were washed and resuspended in PBS and analyzed by flow cytometry.

Cell fractionation and protein extraction. Cells were grown and treated in 35-mm dishes. Fractionation into cytoplasmic and nuclear components was carried out, as described (33). Cells were washed with cold PBS, overlaid with low-salt lysis buffer (20 mM HEPES, 5 mM NaCl, 5 mM $MgCl_2$, 0.5% NP40, 0.1% sodium deoxycholate, 0.05% NaN_3, 1 mM DTT, 50 mg/ml leupeptin, 30 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 10 min. The cells were scraped into microfuge tubes and spun at 1500 rpm for 5 min at 4˚C. The cytoplasmic supernatant was removed and an aliquot of 5X lysis buffer (low-salt lysis buffer with the following modifications: 750 mM NaCl, 2% sodium deoxycholate and 0.5% SDS) was added to adjust the final concentration of the cytoplasmic fraction to 150 mM NaCl, 0.5% sodium deoxycholate and 0.1% SDS.

The nuclear pellet was washed with low-salt lysis buffer and spun for 5 min at 1500 rpm at 4˚C and then lysed in hypertonic lysis buffer (25 mM Tris, 0.5% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing 300 mM NaCl and protease inhibitors. Both fractions were then needle-sheared and pre-cleared by centrifugation for 30 min at 14,000 rpm.

The protein concentration in lysates of both fractions was quantitated spectrofluorometrically with NanoOrange (Molecular Probes, Eugene, OR, USA) or the Bradford assay (Bio-Rad, Hercules, CA, USA) (according to the manufacturer's instructions) using a Gemini microplate reader (Molecular Devices) and SpectraMax spectrophotometer (Molecular Devices) respectively, and then subjected to Western blot analysis as described.

Western blot analysis. Cells were grown to ~70% confluence and either fractioned as described in this section, or total cell lysates were obtained by solubilizing in a NP40-based lysis buffer on ice, followed by cell scraping and clarification by centrifugation at 14,000 rpm for 30 min at 4˚C. Lysates were quantitated for protein by NanoOrange (Molecular Probes) or the Bradford assay (Bio-Rad), and 50 μg of total protein was boiled in SDS Laemmli buffer and separated by SDS-PAGE. The gels were transferred onto PVDF membrane, then blocked with 3% skim milk in TBS for 3 h at room temperature or overnight at 4˚C. The blots were incubated for at least 2 h at room temperature in the appropriate primary antibody. 0.1% Tween 20 and 1% skim milk in TBS were used for washing steps. After several washes, the appropriate alkaline phosphatase-conjugated secondary antibody was added for 1 h. After the final washes, immunoreactive bands were detected by chemiluminescence using the alkaline phosphatase substrate CDP-Star (Roche).

Multiple antigen detection (MAD) immunoblotting. Protein blots were prepared using standard methods. MAD immunoblotting was carried out as described (34). Briefly, after transfer, membranes were washed in PBS and transferred to pre-blocking solution (5% skim milk in TBS 0.1% Tween 20, or 0.2-1% serum from species in which secondary antibody was raised) for 1 h. Blots were incubated overnight in TBS-T containing 0.1% serum from species in which secondary antibody was raised and primary antibody was at an empirically determined appropriate dilution (0.1-1.0 μg/ml for

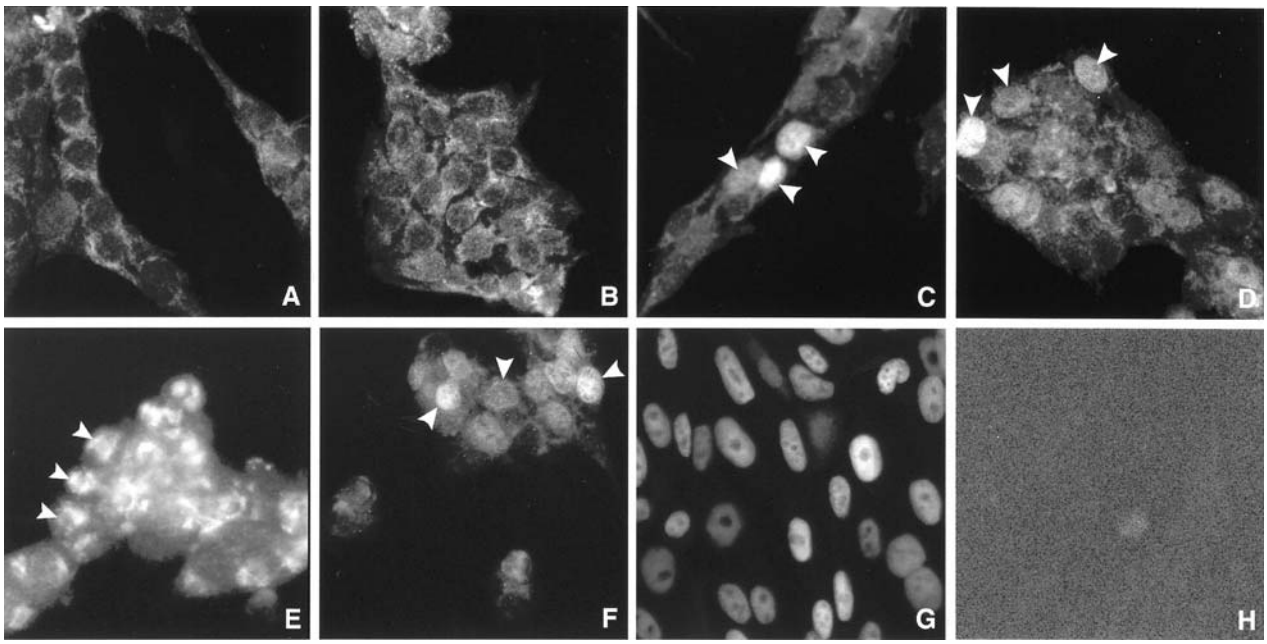

Figure 6. *p53 protein was immunolocalized using a mix of specific monoclonal p53 antibodies (BP53-12 and DO-7) and detected by indirect immunofluorescence. Results were analyzed by confocal microscopy at 40X magnification. (A) Untreated NUB-7 cells demonstrate cytoplasmic p53 staining. (B-E) NUB-7 cells treated with 25 μM curcumin for 1-24 h showed a slight increase in cytoplasmic staining at 1 h (B), apparent nuclear p53 positivity by 3 h (C), prominent p53 nuclear staining concomitant with decrease in cytoplasmic staining at 6 h (D), and apoptotic nuclei positive for p53 after 24 h (E). (F) Treatment with 50 μM resveratrol results in nuclear staining at 12 h. (G) WiT-49 and (H) SAOS-2 served as positive and negative controls, respectively. Arrowheads indicate p53-positive intact nuclei in C, D and F, and apoptotic nuclei in E.*

monoclonals). Blots were rinsed and washed in PBS followed by incubation for 1 h in TBS-T containing 0.2% serum (nonimmune) from species in which secondary antibody was raised, and HRP-conjugated secondary antibody at 1:3000. Blots were rinsed and washed in PBS. Antibody detection was accomplished using a chemiluminescent substrate reagent (SuperSignal Western Blotting Kit, Pierce, Rockford, IL, USA) with exposure to X-ray film, according to the manufacturer's instructions. Blots were then rinsed in PBS and incubated in 0.25 ml/cm^2 Stable DAB chromogenic substrate (Research Genetics, Huntsville, AL, USA) for 20-30 min. Blots were rinsed in tap water to stop the reaction, washed in PBS and then preblocked as above for 15-30 min to prepare for the next cycle of antibody probing.

Immunofluorescence labeling. Cells were grown in 35-mm culture dishes containing glass coverslips (at an initial seeding density of 2x10^5 cells per coverslip) for 2 days. Cells were then treated with 25 μM curcumin for 1-24 h, 50 μM resveratrol for 12 h, 50 μM H7 for 6 h, or left untreated. For p53 localization, cells were rinsed in phosphate-buffered saline (PBS), fixed for 10 min in 10% formalin and washed again in PBS. Cells were permeabilized in 0.5% Triton-X 100 for 15 min and blocked in 10% normal goat serum for 1 h at room temperature. After washing, cells were incubated in 1:100 (in PBS) of the mouse p53 monoclonal PAb-8 overnight at 4˚C, washed and incubated with a 1:100 dilution in PBS of Texas Red-conjugated goat anti-mouse immunoglobulin for 1 h at room temperature followed by further washing. Cells were mounted with Pristine mount (Research Genetics) and examined by fluorescence and confocal microscopy.

Results

The AlamarBlue cytotoxicity assay was used to determine the effect of curcumin and resveratrol on a series of *MYCN*-amplified and overexpressing cell lines representing the most malignant NB phenotype. Treatment of stage 4 NB cell lines with 10-100 μM curcumin and resveratrol for 2-6 days induced morphological features resembling apoptosis, including cell rounding, detachment and fragmentation. Cytotoxicity assays showed a dose-dependent decrease in cell viability with increasing dose and duration of treatment. A dose-dependent cytotoxic effect in NUB-7 and LAN-5 was demonstrated by both compounds after 2 and 6 days of treatment (Figure 1). Curcumin was more potent in inducing cytotoxicity in NB cell lines although both compounds induced >50% cytotoxicity at doses >50 μM when applied for 2 days. When treatment was extended to 6 days (with fresh media and treatment every 2 days), doses as low as 10 μM yielded a significant cytotoxic effect. The latter observation prompted us to further examine the kinetics of the response of NB cell lines to curcumin and resveratrol low-dose treatment administered more frequently within a shorter duration of time. NUB-7 cells were subjected to 0-10 μM curcumin and resveratrol applied

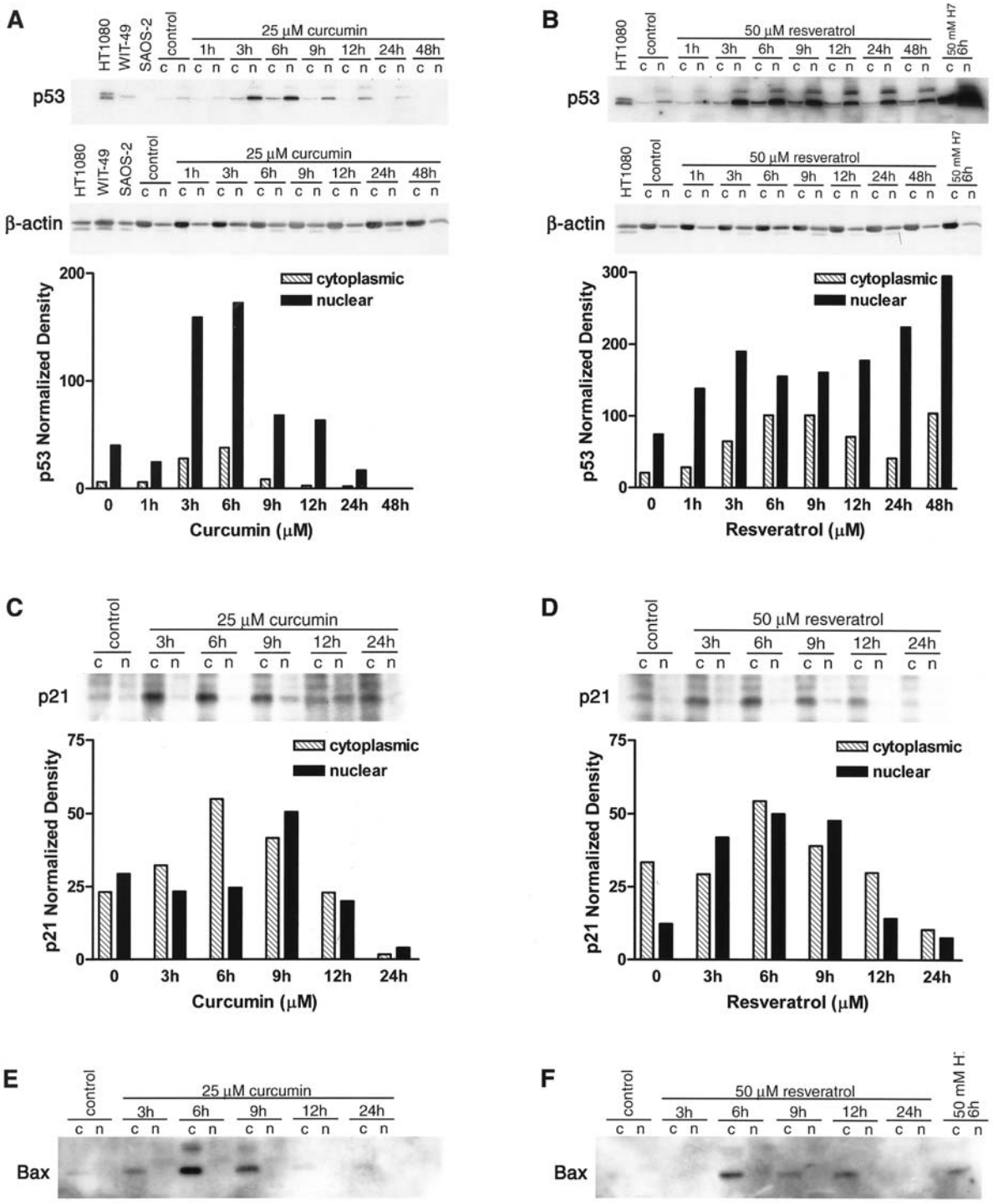

Figure 7. *Curcumin and resveratrol induce nuclear translocation and functional activation of p53 in NUB-7. Cells were treated with 25 μM curcumin or 50 μM resveratrol for 1-48 h and fractioned into cytoplasmic and nuclear fractions. Lysates were quantified and run on SDS-PAGE gel. Western blot was performed to detect the temporal expression and distribution of p53, $p21^{WAF-1/CIP-1}$ and Bax protein in the cellular fractions. Densitometry was performed and results are expressed relative to control untreated cells and in reference to the loading control, β-actin. In A and B, p53 was detected using mouse monoclonal p53 antibodies. Note that p53 accumulated to high levels in the nuclear fractions after 3 and 6 h of curcumin treatment and remained elevated from 3-48 h after resveratrol treatment. In C and D, $p21^{WAF-1/CIP-1}$ was detected with mouse monoclonal $p21^{WAF-1/CIP-1}$ antibody. Both curcumin and resveratrol up-regulated $p21^{WAF-1/CIP-1}$ expression in cytoplasmic and nuclear fractions, coinciding with the pattern of p53 induction and nuclear translocation. In E and F, Bax protein expression was detected using a polyclonal rabbit anti-human Bax antibody. The restricted localization of Bax expression in the cytoplasm also indicates minimal contamination of nuclear fraction with cytoplasmic content. Bax up-regulation occurred coincident with p53 activation but peaked somewhat later.*

every 6 h for 24 h and under these conditions, 10 μM curcumin induced >25% decrease in cell viability. In contrast, 10 μM resveratrol had little effect on NUB-7 viability (Figure 2). To determine the effects of low-dose treatment over a longer time-period, NUB-7 cells were then treated with 0-10 μM of either agent for 8 days. Treatment of NUB-7 with 10 μM curcumin every 2 days for an 8-day period produced >90% decrease in cell number and 5 μM reduced cell viability by >60% over this same period. On the other hand, 10 μM resveratrol reduced cell numbers by only 20% after 8 days of treatment and 5 μM resveratrol had an insignificant effect (Figure 2).

To determine whether this potent effect on NB survival was a common feature of curcumin in NB, we extended the panel of *MYCN*-amplified NB cell lines by including IMR-32 and SK-N-BE(2). Low-dose treatment for 8 days was repeated on the LAN-5, SK-N-BE(2) and IMR-32 cell lines. As shown in Figure 3, 10 μM curcumin reduced cell viability by ~85% in LAN-5, ~80% in IMR-32 and ~50% in SK-N-BE(2). At 5 μM curcumin the order of sensitivity was LAN-5>IMR-32>SK-N-BE(2) with values approaching ~60% decrease in cell viability. In contrast to curcumin, none of these lines were affected by 10 μM resveratrol (not shown).

Since we had initially noted more dramatic responses of NUB-7 and LAN-5 to these compounds at higher doses, we tested whether the other NB cell lines were also sensitive to higher doses of these agents. Examining the effects on IMR-32 and SK-N-BE(2), we found that 25 μM of curcumin and resveratrol produced a significant cytotoxic effect which was greatly increased at the highest doses of 50 μM and 100 μM (Figure 3). Interestingly, SK-N-BE(2) was less sensitive than IMR-32, particularly at the lower doses. Overall, as observed originally on NUB-7 and LAN-5 cell lines, curcumin induced a more potent and rapid cytotoxic effect than resveratrol.

The strong growth inhibitory effect of curcumin and resveratrol at the higher doses was further examined by FACS analysis. Cell cycle profiling revealed that curcumin arrested NUB-7 cells in G1 at 25 μM and in G2/M at 50 μM when cells were treated for 48 h. After 4 days of treatment, the G2/M arrest remained prominent but a greater proportion of cells were now blocked in S-phase at 100 μM. In comparison, curcumin arrested LAN-5 cells in G2/M at 25 μM and in S-phase at 100 μM after 48 h of treatment and predominantly in G2/M after 4 days of treatment. Thus curcumin appeared to mainly produce a cell cycle arrest at the G2/M point in the cell cycle (Figure 4). In contrast to the G2/M arrest induced by curcumin, resveratrol induced a strong S-phase growth arrest in both NUB-7 and LAN-5 with >80% of the cells arresting in cell cycle at 25-50 μM. In parallel with the S-phase arrest, 100 μM resveratrol induced a G1 arrest after 48 h of treatment in NUB-7 and 4 days in LAN-5 (Figure 4). Thus, cell cycle arrest was dose-dependent in G1-, G2/M- and S-phases.

FACS analysis also indicated that at the higher doses, 50 μM and 100 μM, of resveratrol there was a markedly increased subdiploid fraction representing the apoptotic fraction. This was most evident after 4 days of treatment. In fact, 100 μM resveratrol eliminated all viable cells in NUB-7 after 4 days.

Apoptosis of NUB-7 was also examined by TUNEL assay. Figure 5 shows that the percentage of TUNEL-positive cells increased significantly even after 10 μM curcumin treatment for 48 h. The number of TUNEL-positive cells then rose dramatically at 50 and 100 μM curcumin. It is not known whether this high percentage of TUNEL-positive cells is entirely represented in the subdiploid fraction or a proportion remains within the fractions showing growth arrest. Nevertheless, both FACS and TUNEL analyses demonstrate a strong cell cycle and cytotoxic effect of curcumin (and to a lesser degree, resveratrol) on NB cells.

Previous studies had indicated that p53 was sequestered in the cytoplasm of NB cells and was essentially inactive in this context (12, 33). We postulated that p53 could be involved in the observed cytotoxic effects of curcumin and resveratrol since these compounds are capable of inducing cell stresses sensed by p53 (35-37). Functional activation of cytoplasmic p53 leads to nuclear translocation of p53 and transcriptional activation (38). Immunofluorescence labeling of untreated NUB-7 cells showed significant expression of p53 in the cytoplasm with very little detectable in the nucleus (Figure 6). Before testing curcumin and resveratrol for their ability to induce p53 translocation, we treated cells with the general kinase inhibitor H7 which produces a potent apoptotic effect correlated with nuclear translocation of p53 (39). H7 at 50 μM for 6 h induced marked apoptosis of our NB cell lines and induced prominent p53 translocation (not shown). We then treated NUB-7 with 25 μM curcumin for 1-24 h and noted a progressive response in p53 with an apparent increase in cytoplasmic p53 by 3 h, nuclear accumulation by 6 h and apoptotic nuclei positive for p53 after 24 h (Figure 6). Resveratrol treatment at 50 μM for 12 h also resulted in large numbers of cells with nuclear p53 positivity. As positive and negative controls we used a mutant p53-expressing cell line, WiT-49, where all cell nuclei were positive-staining, and SAOS-2, null for p53, showed no staining at all. A similar pattern of p53 nuclear staining was observed in LAN-5 cells treated with curcumin (not shown). Thus curcumin induced a translocation of p53 from cytoplasm to nucleus which was dose- and time-dependent.

To assess the functional outcome of p53 nuclear translocation, we first fractionated cells into cytoplasmic and nuclear fractions and then immunoblotted for p53 expression. The HT1080 and WiT-49 cell lines served as positive controls and SAOS-2 as a p53-negative cell line. On

the basis of the previous result, we selected 25 μM curcumin and 50 μM resveratrol and a 1 h - 48 h treatment range to examine the temporal aspect of p53 expression. Figure 7 shows that by 3-6 h after treatment with 25 μM curcumin there was a dramatic increase in nuclear p53 which then decreased rapidly by 9-12 h after treatment. Treatment with 50 μM resveratrol also induced a rapid nuclear translocation of p53 by 3 h, however p53 nuclear levels remained elevated for up to 48 h. As previously noted (39), H7 produced a potent p53 nuclear translocation after 6 h. Interestingly, with both curcumin and resveratrol the overall level of p53 expression appeared to increase during these periods relative to the control untreated cells and in reference to β-actin, a loading control. Thus treatment with these agents induced a strong p53 response in expression and nuclear translocation and these observations are in parallel to and support the p53 immunolocalization results.

It is well known that p53 activation involves transcriptional up-regulation of several targets including $p21^{WAF-1/CIP-1}$, associated with growth arrest and Bax, associated with induction of apoptosis (40, 41). The rapid increase in p53 protein and its nuclear translocation in our NB cell lines correlated with cell cycle arrest and induction of apoptosis. Thus we examined NB cells for induction of $p21^{WAF-1/CIP-1}$ and Bax in a similar time-dependent manner using the MAD protocol to detect multiple proteins on the same blot. We found that $p21^{WAF-1/CIP-1}$ expression in the cytoplasmic fraction reached a peak at 6-9 h followed by a sharp transient peak at 9 h in the nuclear fraction (Figure 7). In contrast, resveratrol-treated NUB-7 cells showed that nuclear $p21^{WAF-1/CIP-1}$ was markedly increased from 3-9 h in parallel with cytoplasmic $p21^{WAF-1/CIP-1}$. Overall, $p21^{WAF-1/CIP-1}$ expression was induced to a significant level sequentially with the induction of p53 expression and nuclear translocation. A similar pattern of Bax expression was observed in that Bax expression reached a peak at 6 h (Figure 7). Restriction of Bax expression to the cytoplasm was expected as previously described (42). Restricted localization patterns of the different p53 transcriptional targets also served to verify the quality of the nuclear and cytoplasmic preparations. Our analyses indicated minimal contamination of the nuclear fraction with cytoplasmic content.

Discussion

Two chemically diverse phytochemicals, curcumin and resveratrol, with properties as antioxidants and chemopreventives, were evaluated for their effects on human NB cell lines representing the most aggressive variants (43). Here we provide the first evidence of the role of p53 involvement in the cytotoxic effect of these compounds towards malignant neuroblasts cultured *in vitro*. The studies reveal potent growth inhibitory and apoptosis-inducing effects of both compounds on human NB, especially curcumin, which significantly inhibited proliferation in the range of 5-10 μM. Inhibition of tumor cell proliferation and induction of apoptosis occurred in a dose-dependent manner and with different kinetics. Cell fractionation analysis revealed that p53 protein levels were up-regulated in both compartments after treatment with curcumin and resveratrol, however p53 protein was actively translocated to the nuclear compartment within 3-6 h after treatment. Functional activation of p53 was indicated by the up-regulated temporal expression of $p21^{WAF-1/CIP-1}$, accounting for the growth arrest, and of Bax, accounting for the induction of apoptosis, following nuclear translocation of p53. The fact that this occurred in different NB cell lines in spite of the presence of *MYCN* amplification and other pro-malignant genetic changes strongly suggests that exploiting the inherent function of p53 presents a window of opportunity to develop highly effective therapeutics for elimination of NB tumors.

There is controversy over whether nuclear exclusion renders p53 non-functional, with some studies suggesting that it does (12, 13) and others suggesting that it does not (14, 39). Studies by Isaacs *et al.* (13) have shown that different NB subtypes, which coexist in NB cell lines, undergo distinct responses to γ-irradiation such that wild-type p53 function is compromised in N-type cells because of cytoplasmic sequestration, while in S-type cells p53 is not sequestered and exhibits wild-type function. This suggests that the cytoplasmic sequestration of p53 in NB may represent a reversible process (13). However, *in vivo*, N-type cells and also intermediate, or I-type, cells (putative NB tumor stem cells) (44) overwhelmingly constitute the bulk of aggressive, high-stage NB as reflected in the NB cell lines used for the study. It is interesting to note that NUB-7, an I-type NB cell line (27), proved quite sensitive to the anti-tumor effects of curcumin, suggesting that curcumin might hold promise as a chemopreventive for NB.

It is of particular interest that SK-N-BE(2) was the least sensitive of the stage 4 *MYCN*-amplified NB cell lines tested. Unlike the other lines tested which are all wild-type for p53, SK-N-BE(2) harbors a *TP53* mutation that inactivates p53 function (3). It is not surprising then that SK-N-BE(2) was less sensitive than cell lines in which p53 was wild-type and potentially capable of mediating a response to curcumin and resveratrol, indicating that p53 certainly plays a role in mediating the cytotoxic effects of these diphenols. However, SK-N-BE(2) was not completely insensitive to the effects of curcumin and resveratrol. In fact, even at the lower dose of 10 μM curcumin for an extended period of time, SK-N-BE(2) showed a significant loss in viability, suggesting that p53 is not the only mediator of the cytotoxic effects of these diphenols in NB.

Several lines of evidence in the literature strongly suggests that it is the pro-oxidant action of plant-derived polyphenolics rather than their antioxidant activity that may be an important mechanism for their anticancer and apoptosis-inducing properties (45). Of particular interest are studies demonstrating that curcumin (36) and resveratrol (46) cause oxidative strand breakage in DNA in the presence of Cu(II). There is evidence to suggest that cytoplasmic retention of p53 in NB is more pronounced at lower levels of DNA damage and is saturable at severe levels of damage, leading to translocation of p53 to the nucleus followed by wild-type function (41, 47). It is possible then that these agents, particularly at the higher doses, may cause sufficient DNA damage in neuroblastoma to induce nuclear translocation and activation of wild-type p53. However, it is important to note, particularly in the case of curcumin, that 5-10 μM, doses that potentially could be approached *in vivo*, had significant effects. At the lower doses, it is possible that it is not solely the pro-oxidant properties of the polyphenols which are activating p53 and inducing cytotoxic effects, especially considering that other mechanisms may also be mediating the anti-tumor effects of curcumin and resveratrol.

From a clinical perspective, combining dietary polyphenols and the addition of other phytochemicals that enhance bioavailability could improve the therapeutic efficacy of polyphenols (48, 49). It is conceivable that therapeutic regimens incorporating curcumin and resveratrol as adjuncts could take advantage of individual differences and potencies, as noted here for curcumin and resveratrol, to maximize their therapeutic potential. Combinations of phytochemicals might possess enhanced activity against cancers, although in specific cases the properties of phytochemicals may interfere with the activities of certain chemotherapeutic agents. For example, one study showed that resveratrol blocks paclitaxel-induced cell death in NB (50), while another showed that dietary supplementation with curcumin was found to significantly inhibit cyclophosphamide-induced apoptosis and tumor regression (51).

Nevertheless, despite certain limitations, these same phytochemicals have shown potent activity against established tumors with negligible toxicity to normal cells, and possess potential for clinical use. Whether as primary chemotherapeutics or adjuncts, given their anti-tumor potential, they may be optimally used in a schedule alternating with chemotherapy, or prior to therapy as chemosensitizing agents for tumors with a resistant phenotype, and as prophylactics post-chemotherapy against tumor recurrence and metastasis. Therefore, given the poor outcomes of current chemotherapy in the treatment of advanced stage NB, the present findings and the innate properties of these agents, should encourage further preclinical studies and possibly their eventual incorporation into treatment protocols. If proven effective in a severe test model like neuroblastoma, phytochemicals could dramatically lower the tumor burden in the pediatric population.

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