

## Resveratrol Inhibits Tumor Growth of Human Neuroblastoma and Mediates Apoptosis by Directly Targeting Mitochondria

Paul R. van Ginkel,<sup>1</sup> Dhruv Sareen,<sup>1,2</sup> Lalita Subramanian,<sup>1</sup> Quintisha Walker,<sup>1</sup> Soesiawati R. Darjatmoko,<sup>1</sup> Mary J. Lindstrom,<sup>3</sup> Amol Kulkarni,<sup>1</sup> Daniel M. Albert,<sup>1,4</sup> and Arthur S. Polans<sup>1,2,4</sup>

**Abstract Purpose:** Neuroblastoma is an aggressive childhood disease of the sympathetic nervous system. Treatments are often ineffective and have serious side effects. Because resveratrol, a natural plant product, has been reported to have limited toxicity at chemotherapeutic levels, we investigated its efficacy in the treatment of neuroblastoma as well as its underlying mechanism of action.

**Experimental Design:** Resveratrol was tested in mouse xenograft models of human neuroblastoma and *in vitro* using human cell lines.

**Results:** Resveratrol inhibited the outgrowth of tumors by as much as 80%. The bioavailability of the drug in serum was in the low micromolar range (2-10  $\mu\text{mol/L}$ ) and no accumulation was observed in tumor tissue. When resveratrol levels were increased by peritumor injection, rapid tumor regression occurred. Resveratrol decreased tumor cell viability *in vitro* by 75% to 90%, resulting from an inhibition of cell proliferation and an induction of apoptosis. Loss of mitochondrial membrane potential was an early response to resveratrol. In addition, resveratrol treatment of isolated mitochondria also led to depolarization, suggesting that the drug may target mitochondria directly. Following depolarization, resveratrol caused the release of cytochrome *c* and Smac/Diablo from the mitochondria and subsequently the activation of caspase-9 (4- to 8-fold) and caspase-3 (4- to 6-fold).

**Conclusions:** These studies indicate that, despite low bioavailability, resveratrol is effective at inhibiting tumor growth. Elevated levels of resveratrol enhance its antitumor potency leading to tumor regression, associated with widespread tumor cell death, the underlying mechanism of which involves the direct activation of the mitochondrial intrinsic apoptotic pathway.

Neuroblastoma is the most common cancer during infancy and the most common solid extracranial cancer of childhood in the United States, with an annual incidence of  $\sim 9.1$  cases per million children under the age of 15 (1). At the time of diagnosis,  $\sim 70\%$  of patients present with distant metastases. Standard treatment involves radiation and chemotherapy. Chemotherapeutic agents include cisplatin, doxorubicin, etoposide, and cyclophosphamide. However, the side effects of these treatments in children can be serious due to both acute damage and toxicity and increased occurrence of secondary

tumors. In addition, even with aggressive treatment, mortality is still high in more advanced stages of the disease with  $<50\%$  survival rate (2). Therefore, the search for new nontoxic drugs for single or multidrug therapy is especially important.

There is significant interest in natural products that have clinical potential in the prevention and treatment of cancer. One product that has shown considerable promise is resveratrol, 3,5,4'-trihydroxy-*trans*-stilbene, a phytoalexin found in grapes, berries, and peanuts and present in the human diet. First described by Jang et al. (3) to have chemopreventive activity, it has subsequently been shown to inhibit the growth of and induce apoptosis in many different tumor cells *in vitro* as well as inhibit tumor growth in a few animal models (4). In spite of these findings, little work has been done about the effect of resveratrol in neuroblastoma (5). Using a murine neuroblastoma cell line, *in vitro* studies showed a cytotoxic effect of resveratrol, and animal studies using the same mouse cell line showed inhibition of tumor growth (6). Unfortunately, preclinical *in vivo* studies of the human disease are lacking.

Resveratrol has pleiotropic effects, altering many different signaling pathways (i.e., nuclear factor- $\kappa\text{B}$ , Rb-E2F, p53, phosphatidylinositol 3-kinase/Akt, and mitogen-activated protein kinase pathways), leading to suppression of tumor cell proliferation, adhesion, invasion and metastasis, reduced signs of inflammation and angiogenesis, and induction of apoptosis and differentiation (4). Although numerous studies have described intracellular changes leading to cell cycle arrest or

**Authors' Affiliations:** Departments of <sup>1</sup>Ophthalmology and Visual Sciences, <sup>2</sup>Biomolecular Chemistry, and <sup>3</sup>Biostatistics and Medical Informatics, University of Wisconsin School of Medicine and Public Health; and <sup>4</sup>University of Wisconsin Paul P. Carbone Comprehensive Cancer Center, Madison, Wisconsin  
Received 2/13/07; revised 6/13/07; accepted 6/28/07.

**Grant support:** NIH grant R01CA103653 (A.S. Polans), NASA Sharp Program (Q. Walker), Retina Research Foundation, Mandelbaum Cancer Therapeutics Initiative (A.S. Polans and D.M. Albert), and NIH core grant P30 EY016665-02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Requests for reprints:** Arthur S. Polans, Department of Ophthalmology and Visual Sciences, University of Wisconsin School of Medicine and Public Health, Room K6/466 Clinical Sciences Center, 600 Highland Avenue, Madison, WI 53792. Phone: 608-265-4423; Fax: 608-265-6021; E-mail: aspolans@wisc.edu.

©2007 American Association for Cancer Research.  
doi:10.1158/1078-0432.CCR-07-0347

apoptosis in response to resveratrol treatment, the effects are often cell type specific, and these studies have not yet identified the underlying mechanism of drug action (4, 7). Wang et al. (8) described the use of a resveratrol affinity column, which identified NQO2 (NRH:quinone reductase type 2) as one of the targets of resveratrol in K562 leukemia cells. However, it is not clear how NQO2 inhibition might lead to apoptosis in cancer cells. More recently, the  $\alpha_v\beta_3$  receptor was described as a potential target for resveratrol in MCF-7 cells, leading to apoptosis via extracellular signal-regulated kinase and p53 signaling (9). Alternatively, mitochondrial involvement in resveratrol-induced cell death has been described (10–12).

Mitochondrial dysfunction is an important signal for apoptosis. In cells that die by signaling via the intrinsic apoptotic pathway, mitochondria play a central role. Loss of membrane potential can signal apoptosis by the release of cytochrome *c* and Smac/Diablo from mitochondria and a decrease in the Bcl-2-Bax ratio associated with mitochondria, leading to the activation of caspase-9 via APAF-1 and to the activation of caspase-3 (see ref. 13 for review).

Here, we show that the loss of mitochondrial membrane potential is an early resveratrol-induced event in neuroblastoma cells that can occur as a result of direct targeting of the mitochondria by the drug. Resveratrol treatment results in the release of cytochrome *c* and Smac/Diablo and the activation of subsequent components of the mitochondrial intrinsic apoptotic pathway and eventually tumor cell death *in vitro*. We show that resveratrol given by oral gavage significantly diminishes tumor growth in two xenograft models of human neuroblastoma. Furthermore, we find a sharp increase in tumor cell death and tumor regression when resveratrol is injected in the vicinity of the tumor. Owing to its nontoxic characteristics coupled with the frequency of secondary cancers in children treated with conventional chemotherapeutic agents, resveratrol is an excellent candidate for clinical trials of neuroblastoma.

## Materials and Methods

**Cell culture.** SK-N-AS, NGP, and SH-SY5Y neuroblastoma cell lines were grown as adherent cells at 37°C, 5% CO<sub>2</sub> in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals), 10 mmol/L HEPES, and 1% penicillin-streptomycin-amphotericin B (Sigma).

**Inoculation of mice.** NGP cells ( $1 \times 10^6$ ) were mixed 1:1 with Matrigel (BD Biosciences) and injected s.c. into the flanks of 60 athymic mice (Harlan), which were subsequently divided into four groups of 15 animals each. Protocols were approved by the University of Wisconsin-Madison Research Animals Resources Center. Five days following inoculation, mice were given 2, 10, and 50 mg/kg of resveratrol (Cayman Chemical) in Neobee M5 oil (triglyceride of coconut oil purchased from Spectrum Chemical Manufacturing Corp.) or Neobee M5 oil alone by oral gavage daily for 5 weeks and the growth rate of s.c. tumors was monitored. Fifty milligram per kilogram was chosen as the maximum dose because the drug no longer stays in solution in Neobee oil at higher doses. SK-N-AS cells ( $1 \times 10^6$ ) were injected s.c. into 30 athymic mice, divided into groups of 10 mice, and given 50 mg/kg resveratrol in Neobee M5 oil or Neobee M5 oil alone or left untreated for 5 weeks. Tumor size in the animals was measured biweekly with calipers in three dimensions (length, width, and height) measuring to the nearest millimeter, and the volume was determined by multiplying the three measurements. A final measurement was made after the tumor was taken out of the animal.

**Peritumor injections.** SK-N-AS cells ( $3 \times 10^6$ ) were injected s.c. in 16 athymic mice and tumors were allowed to grow to  $\sim 200$  mm<sup>3</sup>. The mice were divided into four groups of four animals each and given five injections over 16 days of 5, 10, and 20 mg of resveratrol in 200  $\mu$ L DMSO or DMSO alone. The animals were euthanized and photographed and tumor tissue was fixed in formalin, sectioned, and stained with H&E.

**Statistical analysis.** All responses, tumor volume, relative fluorescence units, percentage apoptotic cells, JC-1 red/green ratio, JC-1 red fluorescence intensity, and caspase-9 and caspase-3 activation, raw numbers not proportions, were analyzed using one-way ANOVA. If this initial analysis found a significant difference among the groups, then *t* tests were used to test for pairwise differences between groups. All responses except caspase activation were transformed to the log scale to obtain data, which satisfy the assumptions required for ANOVA. Means and SEs were calculated by transforming back from the log scale. Differences were considered significant at  $P < 0.05$ .

**Resveratrol bioavailability.** Resveratrol measurements were carried out using standard procedures (14). Briefly, tissues or organs were frozen in liquid nitrogen, powdered in a mortar, and homogenized in sodium acetate [0.05 mol/L (pH 5.0); 100 mg powder/0.1 mL]. Samples (tissue homogenates or serum) were incubated at 37°C for 5 h in the presence of 10,000 units/mL  $\beta$ -glucuronidase (Sigma) and then extracted with  $2 \times 0.5$  mL ethyl acetate. Upper phases were mixed and the solvent was evaporated. The residual pellets were dissolved in 0.1 mL high-performance liquid chromatography solvent B (0.09% trifluoroacetic acid in 90% acetonitrile-water).

Samples were analyzed by reverse-phase high-performance liquid chromatography using a Gemini C6-phenyl column ( $4.5 \times 250$  mm) with 5- $\mu$ m particle size (Phenomenex). The mobile phase consisted of a 40:60 mixture of solvent A (0.1% trifluoroacetic acid in water) and solvent B pumped at 1 mL/min. The eluent was monitored at 305 nm. Calibration curves were obtained using resveratrol standard solutions (0–2,000 pmol) and found to be linear with a correlation coefficient  $>0.99$ . As an additional control, resveratrol-spiked serum was analyzed and found to be extracted with a 90% efficiency.

**Tumor cell viability.** Neuroblastoma cells were grown in 96-well microtiter plates for 2 days. Resveratrol was added, and 1 to 8 days later, CellTiter-Blue reagent was added according to the manufacturer's instructions (Promega). Fluorescence was measured at excitation/emission wavelengths of 560/590 nm using a fluorescence plate reader (Molecular Devices).

**Flow cytometry.** Cells were treated for 48 h with resveratrol and nuclei were then prepared for flow cytometry as described (15). Nuclei were analyzed in a flow cytometer by collecting 20,000 events.

**Apoptosis determinations.** SK-N-AS, NGP, and SH-SY5Y cells were treated with 200, 100, and 50  $\mu$ mol/L of resveratrol, respectively. Cells were then fixed, stained with Hoechst 33528 (Molecular Probes), and analyzed as described (15). The percentage of apoptotic cells was calculated as the number of apoptotic cells relative to the total number of cells in a viewing field and averaged over five different fields.

**Isolation of mitochondria.** Mitochondria were isolated from  $5 \times 10^7$  cells as described earlier (15).

**Mitochondrial membrane potential.** Changes in mitochondrial membrane potential in whole cells and in isolated mitochondria were measured by fluorometry using the JC-1 dye (Cell Technology) as described (15).

**Cytochrome *c* and Smac/Diablo release.** Cytochrome *c* and Smac/Diablo were compared by immunostaining in cytosolic fractions from  $6 \times 10^6$  cells either treated with 100  $\mu$ mol/L resveratrol or left untreated. Cell fractionation was achieved as described earlier (15). In experiments using isolated mitochondria from  $5 \times 10^7$  cells, cytochrome *c* levels were compared in mitochondria and supernatant of drug-treated and untreated mitochondria by Western blot analysis. Protein concentration was measured using the Bio-Rad protein assay. Western blot analysis was carried out using 15% SDS-polyacrylamide gels and either anti-cytochrome *c* (BD PharMingen) or anti-Smac/Diablo (Cell Signaling

Technology) antibodies. Purity of the cytoplasmic and mitochondrial fractions was ascertained with anti-glyceraldehyde-3-phosphate dehydrogenase and anti-cytochrome oxidase antibodies.

**Caspase activity.** Cell lysates were prepared from resveratrol-treated and untreated neuroblastoma cells to measure the activity of caspase-3 and caspase-9 by fluorometry. SK-N-AS, NGP, and SH-SY5Y cells were treated with 10, 50, 100, and 200  $\mu\text{mol/L}$  of resveratrol. Cells were lysed in a buffer containing 5 mmol/L HEPES and 0.05% Triton X-100. Total protein concentration was measured by Bradford assay (Bio-Rad). For caspase-9 activation, 100  $\mu\text{g}$  of total protein and, for caspase-3 activation, 20  $\mu\text{g}$  of total protein from each lysate were assayed in caspase reaction buffer (20 mmol/L HEPES, 40% glycerol, 10 mmol/L DTT, 1 mmol/L EDTA) as described earlier (15). Caspase-8 activation was measured by Western blot analysis using an anti-caspase-8 antibody (Cell Signaling Technology).

## Results

To determine if resveratrol is effective in inhibiting neuroblastoma tumor growth, mouse xenograft models of human neuroblastoma cell lines were tested. Initially, a dose-response experiment was done with the NGP xenograft model. Mice were treated daily with resveratrol doses of 2, 10, and 50 mg/kg. After ~5 weeks of oral drug treatment, tumor growth was most strongly inhibited at the highest dose and proportionately less with lower doses compared with the control group (Table 1A). Tumor volume ranged from ~70% less in the 50 mg/kg-treated group to 56% less in the 2 mg/kg-treated group compared with the control group ( $P < 0.0001$  for both). Growth kinetic data show that significant differences between the treated and untreated tumors arose as soon as exponential tumor growth started at around day 15 (Fig. 1A).

Because the highest dose was most effective in inhibiting tumor growth in the NGP xenograft model, it was also used for the SK-N-AS xenograft model. After 5 weeks of oral drug treatment, tumor growth was strongly inhibited compared with the control groups that received vehicle alone or were left untreated (Table 1B). Tumor volume was ~80% less than that in the vehicle-treated and untreated control groups ( $P = 0.0003$  and 0.0006, respectively). Tumor growth kinetic data show that this tumor displayed extensive growth after a 3-week lag and

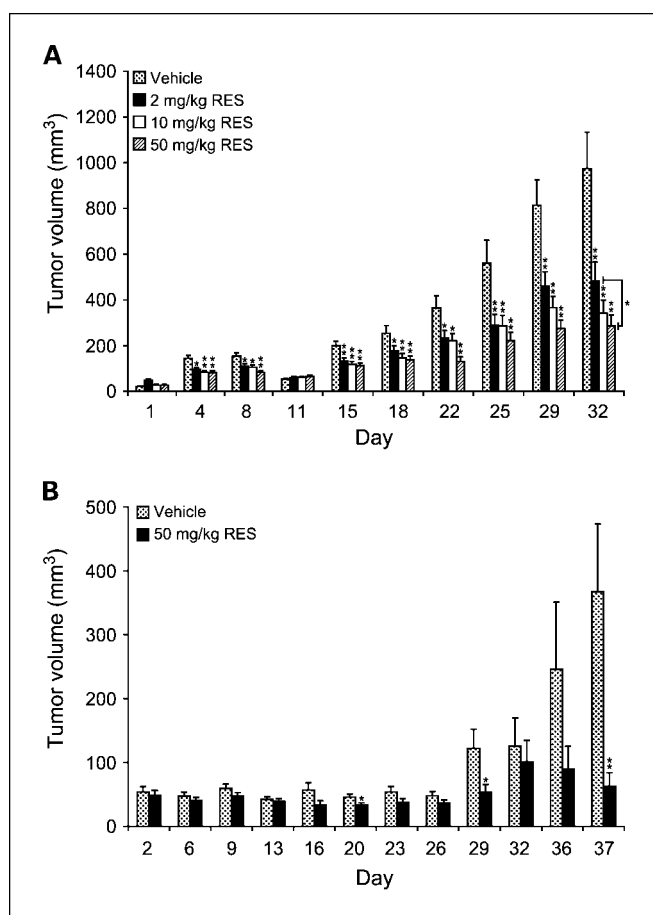
**Table 1.** Inhibition of neuroblastoma tumor growth

<b>A</b>			
	Tumor size ( $\text{mm}^3$ )	SE	$P^*$
50 mg/kg resveratrol	274.04	39.50	<0.0001
10 mg/kg resveratrol	307.85	63.91	<0.0001
2 mg/kg resveratrol	434.05	71.09	<0.0001
Vehicle	982.27	115.66	
<b>B</b>			
	Tumor size ( $\text{mm}^3$ )	SE	$P^\dagger$
50 mg/kg resveratrol	62.99	21.36	
Vehicle	368.38	106.18	0.0003
Untreated group	313.14	132.77	0.0006

NOTE: End tumor volumes after 5 wks of oral resveratrol treatment in NGP (A) and SK-N-AS (B) xenograft models.

\* $P$  value when compared to the control group.

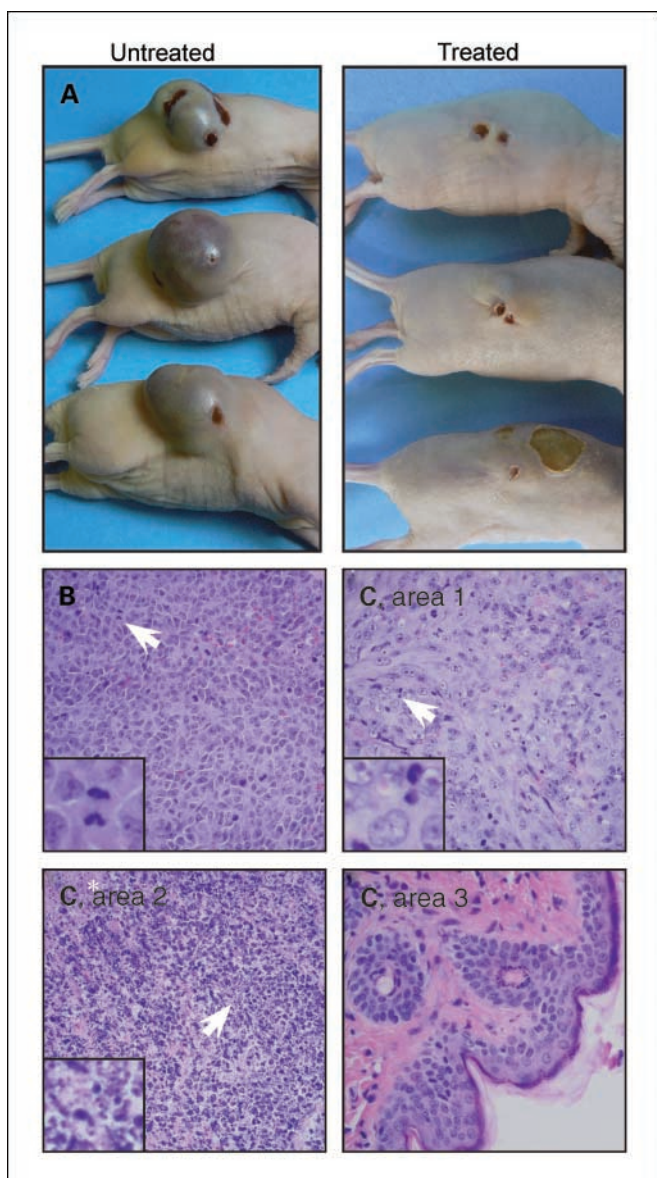
$^\dagger P$  value when compared to the treated group.



**Fig. 1.** Inhibition of neuroblastoma tumor growth. Tumor growth kinetics during resveratrol (RES) treatment course of NGP (A) and SK-N-AS (B) xenograft models by oral gavage. Plotted is the average tumor growth in resveratrol-treated and vehicle-treated groups of mice. \*\*,  $P < 0.0005$ ; \*,  $P < 0.05$ .

that resveratrol significantly inhibited this growth toward the end of the 5-week treatment (Fig. 1B). Toxicity was assessed by survival, activity, and, twice weekly, body weight. All animals survived the treatment without evidence of toxic effects and animals gained 10% to 15% of weight compared with their pretreatment values. On autopsy, the major organs showed no signs of toxicity.

Despite the inhibition of tumor growth observed in the xenograft models, resveratrol levels 30 min after oral administration of 50 mg/kg of drug were only 2 to 10  $\mu\text{mol/L}$  in serum and about 2 to 10 nmol/g in the liver and decreased thereafter. When drug levels were measured at the end of the 5-week time course, we found no accumulation of resveratrol in the serum, liver, or tumor tissue, with levels in the tumor being comparable with those in the liver (about 2-10 nmol/g). To assess the effect of higher doses of resveratrol on tumor growth and viability, the drug was injected next to the tumor in a series of five injections. This resulted in rapid tumor regression compared with the vehicle-treated controls (Fig. 2A). Vehicle-treated tumor tissue shows the presence of healthy tumor cells with clear borders and the presence of many mitotic figures (Fig. 2B). In the resveratrol-treated tumors, there are signs of early necrosis and increased presence of apoptotic cells interspersed with residual surviving tumor cells. Cell borders



**Fig. 2.** Tumor regression after peritumor injection of resveratrol in the SK-N-AS xenograft model. *A*, tumor size after five injections of vehicle or 5 mg resveratrol. H&E-stained sections of vehicle-treated tumor (*B*), 5 mg resveratrol-treated tumor (*C, area 1*), and 10 mg resveratrol-treated tumor (*C, area 2*) and adjacent normal skin tissue in the 5 mg – treated condition (*C, area 3*). Insets show area around arrow in detail. Inset in (*B*), mitotic body. Insets in (*C*), areas 1 and 2, apoptotic cells. Asterisk in (*C*), area 2, necrotic area.

and nuclear detail are less clear, and together with cytoplasmic swelling, these changes are consistent with cell damage (Fig. 2*C, area 1*). Other areas show many pyknotic nuclei suggestive of apoptosis and surrounded by areas of necrosis (Fig. 2*C, area 2*). Adjacent normal tissue is relatively unaffected (Fig. 2*C, area 3*).

To understand the mechanism of the antitumor activity of the drug, *in vitro* studies were carried out using the SK-N-AS, NGP, and SH-SY5Y cell lines, which differ in their genetic abnormalities representing some of the major subtypes of neuroblastoma (16). The viability of all three cell lines was sharply decreased in response to resveratrol treatment in a time- and concentration-dependent manner (Fig. 3*A*). At the highest

drug concentration, cell viability decreased approximately 85% to 90% compared with untreated cells after 5 days of treatment. In the cell lines, the  $IC_{50}$ s of resveratrol after 48 h of treatment were as follows: SK-N-AS, 70  $\mu$ mol/L; NGP, 120  $\mu$ mol/L; and SH-SY5Y, 100  $\mu$ mol/L. If treatment was continued, all cells were dead after approximately 8 to 10 days (data not shown). In addition, dense cultures of neuroblastoma cells (>90% confluent) were also treated with resveratrol as a function of time and concentration and their viability was measured. Under these conditions, cell viability decreased in a concentration-dependent manner over 5 days, further indicating the occurrence of cell death (Fig. 3*B*).

To determine if resveratrol causes an inhibition of cell proliferation, neuroblastoma cells were analyzed by flow cytometry (Fig. 3*C*). Treatment of SK-N-AS cells with resveratrol led to an increase in the percentage of cells in  $G_0$ - $G_1$  phase (34-43%) and S phase (24-30%) and a concurrent decrease in the percentage of cells in  $G_2$ -M phase (42-27%), suggesting arrests in  $G_1$  and S phases of the cell cycle. NGP cells showed an accumulation of cells in S phase (22-64%) and concurrent decrease in the percentage of cells in  $G_0$ - $G_1$  phase (71-36%), suggesting an arrest in S phase. These results indicate that resveratrol decreases cell viability at least in part due to interference with cell cycle progression, although the point of arrest seems cell type specific and dependent on the concentration of resveratrol as seen in other studies (4, 17, 18).

To determine if resveratrol treatment results in enhanced apoptosis, neuroblastoma cells were treated with different concentrations of resveratrol, optimized for maximum apoptotic response over 48 h, and then stained with Hoechst 33528 dye to visualize apoptotic nuclear morphology (Fig. 3*D*). The percentage of apoptotic SK-N-AS and SH-SY5Y cells steadily increased over 48 h up to 32% and 21% of apoptotic cells, respectively. The fraction of apoptotic NGP cells reached a maximum at 24 h and then leveled off. These results suggest that, in addition to the effect of resveratrol on proliferation, the decrease in cell viability is also due to an increase in apoptosis.

Because resveratrol treatment of tumor cells resulted in ultrastructural damage of the mitochondria before cell death,<sup>5</sup> the intrinsic apoptotic pathway in which the mitochondria play a central role was studied in neuroblastoma cells. Mitochondrial membrane potential was first measured as an indicator of mitochondrial dysfunction in SK-N-AS, NGP, and SH-SY5Y cells (Fig. 4*A*). These cell lines showed an early and sustained loss of mitochondrial membrane potential in response to resveratrol treatment. Increasing concentrations of the drug led to increased loss of membrane potential as measured with JC-1. Both resveratrol and the positive control (FCCP) caused a collapse of the membrane potential as indicated by the sharp decrease in the red-green JC-1 fluorescence ratio. These results suggest a mechanism for resveratrol action involving mitochondrial function leading to apoptosis via the intrinsic pathway.

To test whether this involves direct targeting of this organelle, mitochondria were isolated from these cells and treated with resveratrol, and the mitochondrial membrane potential was measured (Fig. 4*B-D*). Because no cytoplasmic compartment is present in these experiments, only the red fluorescence at

<sup>5</sup> Unpublished observations.

595 nm was measured. As an additional positive control, mitochondria were treated with sodium azide. Membrane potential of isolated mitochondria decreased after resveratrol treatment in a dose-dependent manner, suggesting that resveratrol causes mitochondrial dysfunction by directly targeting the mitochondria without the need to interact with cytosolic components. As expected, the drug concentration needed to attain these effects was about 10- to 20-fold less than needed for whole cells probably due to the lack of diffusion barriers.

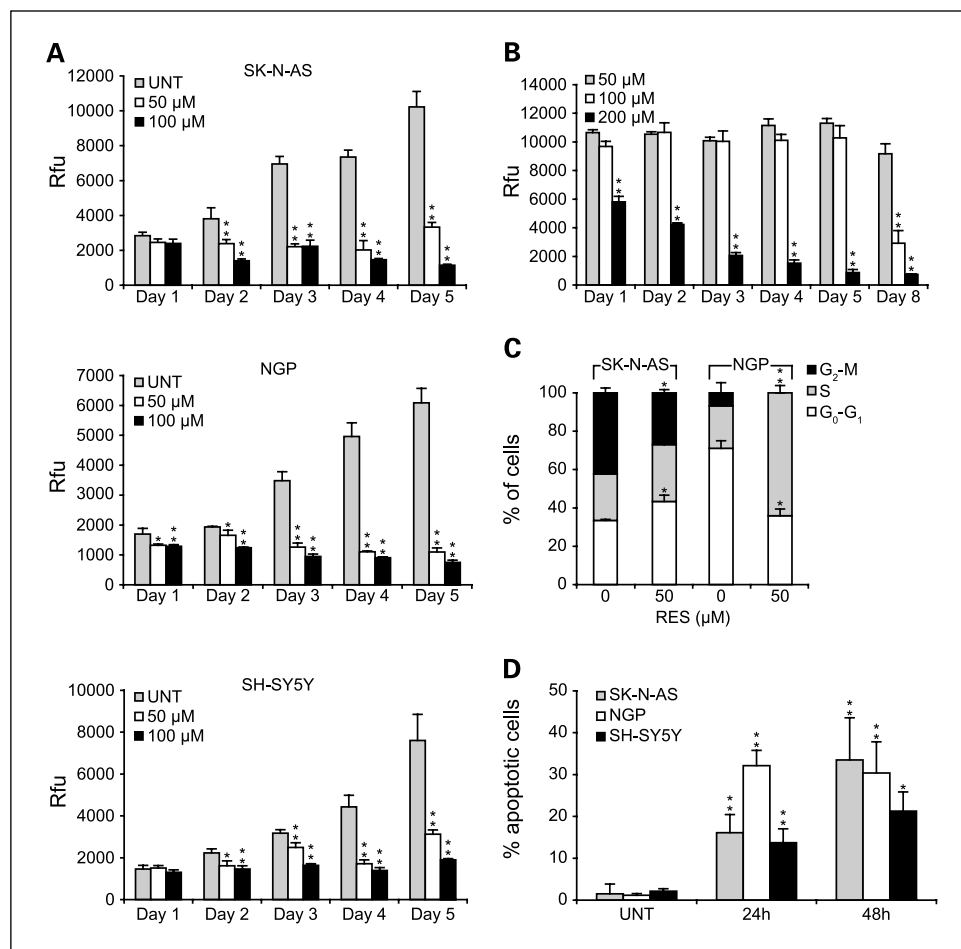
In other cell types, loss of mitochondrial membrane potential is followed by release of cytochrome *c* and Smac/Diablo from the mitochondria (13). To determine if this occurs in neuroblastoma cells, SK-N-AS, NGP, and SH-SY5Y cells were treated with resveratrol, lysed, and fractionated, and the amount of cytochrome *c* and Smac/Diablo found in the cytosolic fraction was measured by Western blot analysis. Cytochrome *c* was released into the cytosol in all three cell types, albeit at different times after initiating drug treatment (Fig. 5A). In SK-N-AS cells, cytochrome *c* was already detectable in the cytosolic fraction after 2 h, whereas in NGP and SH-SY5Y release occurred after 24 to 48 h. Smac/Diablo was also released from the mitochondria in all three cell lines.

To test if release occurs from isolated mitochondria, mitochondria from SK-N-AS cells were treated with 100  $\mu\text{mol/L}$  resveratrol and cytochrome *c* release was measured in the supernatant by Western blot analysis. After 3 h of drug treatment, no cytochrome

*c* release was detectable, suggesting that cytochrome *c* release requires cytoplasmic components to occur. It has been shown that Bax can mediate cytochrome *c* release by translocating to the mitochondria where it heterodimerizes to form channels in the outer membrane (19). Therefore, the lack of cytochrome *c* release from isolated mitochondria may be due to the absence of Bax. Because cells in all three lines undergo mitochondrial membrane depolarization within 15 min of resveratrol treatment, differences in the timing of cytochrome *c* release may be due to these cytoplasmic factors.

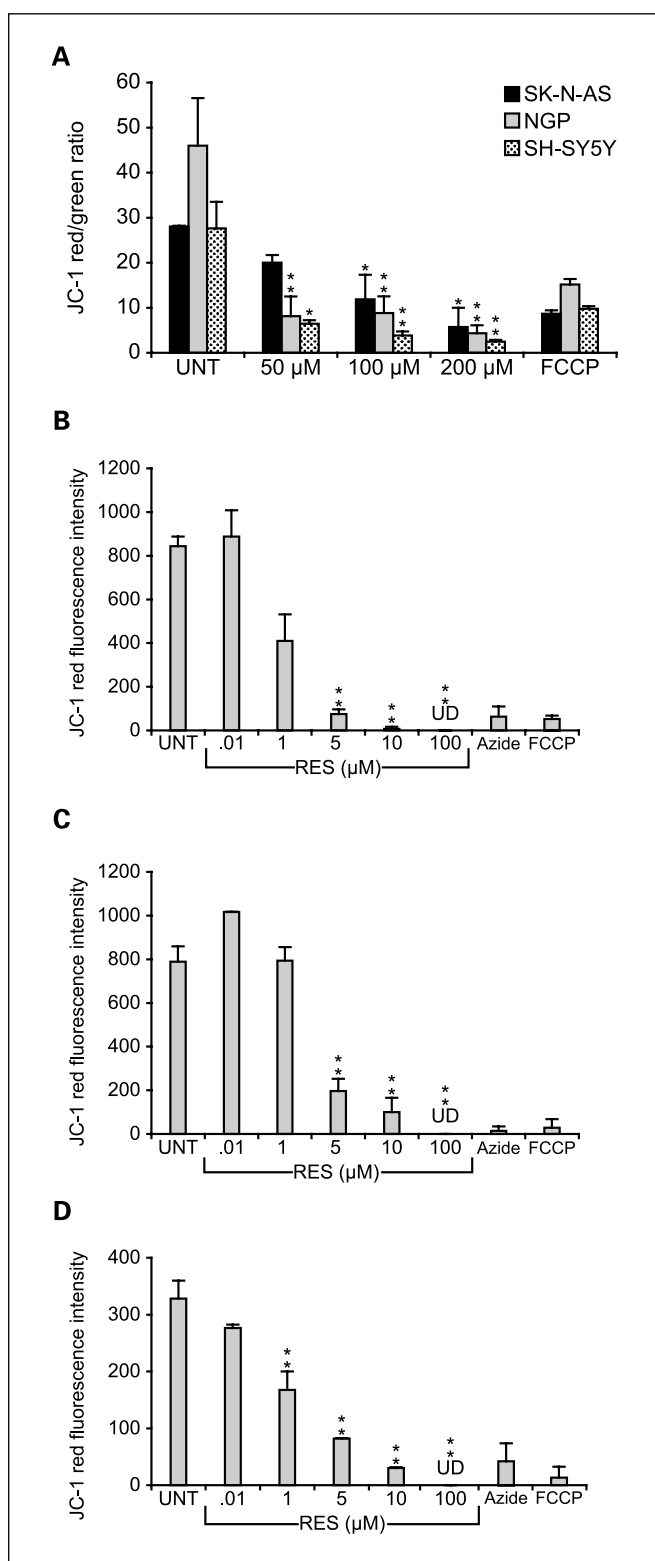
To determine whether other components of the mitochondrial cell death pathway were activated, caspase-9 and caspase-3 activities were measured in resveratrol-treated cells in a dose-response experiment (Fig. 5B and C). All three cell lines showed increased activity of these enzymes with increasing drug concentration, suggesting that these downstream components in the intrinsic apoptotic pathway are activated in response to resveratrol treatment.

Although the data presented above indicate direct targeting of mitochondria by resveratrol, the pleiotropic effects of the drug may also lead to activation of other cell death pathways. A major initiator caspase acting upstream in both chemical-induced apoptosis (20) as well as in the receptor-mediated (extrinsic) apoptotic pathway is caspase-8. To determine if either of these pathways is activated in response to resveratrol treatment, processing of procaspase-8 was measured. No



**Fig. 3.** Resveratrol decreases cell viability of neuroblastoma cell lines by inhibiting cell cycle progression and inducing apoptosis. Cell viability of SK-N-AS, NGP, and SH-SY5Y (A) cells was measured as a function of resveratrol concentration and time after drug addition and plotted as relative fluorescence units (*Rfu*). Dose (>90% confluent) NGP cell viability (B) was measured similarly. Each experiment was done in triplicate. Cell cycle distribution after 48 h of resveratrol treatment of SK-N-AS and NGP cells (C) was measured by flow cytometry using propidium iodide and plotted as the percentage of cells in different phases of the cell cycle. The experiment was done in duplicate. D, apoptotic nuclei of SK-N-AS, NGP, and SH-SY5Y cells were visualized in resveratrol time courses with Hoechst 33258 dye and plotted as the percentage of apoptotic cells relative to the total number of cells. Cell numbers were counted in five different viewing areas under a fluorescence microscope and averaged. UNT, untreated group. \*\*,  $P < 0.0005$ ; \*,  $P < 0.05$ .

Downloaded from http://aacrjournals.org/clinccancerres/article-pdf/13/17/5162/2296024/5162.pdf by guest on 13 June 2024



**Fig. 4.** Resveratrol causes loss of mitochondrial membrane potential in neuroblastoma cells and in isolated mitochondria. Mitochondrial membrane potential in SK-N-AS (A and B), NGP (A and C), and SH-SY5Y (A and D) cells and mitochondria, respectively, was measured as a function of resveratrol concentration 15 min after drug addition using JC-1 dye. As a positive control, cells were treated with the protonophore FCCP or sodium azide. Data were plotted as the ratio of red and green fluorescence intensity at 530/590 nm, respectively, in cells and as the red fluorescence intensity in isolated mitochondria. Each experiment was done in duplicate. UD, undetectable. \*\*,  $P < 0.0005$ ; \*,  $P < 0.05$ .

caspace-8 was detectable in NGP cells (Fig. 5D). Similarly, it has been reported that caspase-8 is absent from SH-SY5Y cells (21). Procaspase-8 was cleaved in SK-N-AS cells in a time-dependent manner indicated by the appearance of 43- and 41-kDa intermediate cleavage products and an 18-kDa active product (Fig. 5D). These three cell lines, however, are equally sensitive to resveratrol treatment *in vitro*. Because most studies suggest that caspase-8 is an essential part of the extrinsic pathway, these data indicate that this pathway does not have a critical role in mediating the apoptotic response to resveratrol.

## Discussion

Neuroblastoma is a childhood cancer that is difficult to treat especially in advanced stages, and current treatments can have severe side effects. Therefore, the search for effective and nontoxic drugs is critical. A requirement for clinical studies is to show that the drug is effective in neuroblastomas of different genetic origins. Lastowska et al. (16) identified different types of neuroblastoma tumors, of which the advanced types fall into three major genetic groups: those with a gain of chromosome 17q, those with a deletion of chromosome 1p, and those with amplification of N-myc. NGP cells have all three of these traits, and this subtype is considered the most malignant. SK-N-AS cells have a gain of 17q, deletion of 1p, but no amplification of N-myc, and SH-SY5Y cells only have a gain of 17q. In the current study, resveratrol was tested in mouse models of two of the more malignant subtypes. Our studies show a very potent inhibitory effect of resveratrol on tumor growth in these animal models of human neuroblastoma. After 5 weeks of treatment, average tumor volume in the animal models is 70% to 80% less than that of the control groups. Because drug treatment in these experiments commenced 5 days after inoculation of the tumor cells when tumor size is minimal, these experiments suggest that resveratrol can prevent outgrowth of small tumors. In addition, in SK-N-AS xenograft mice, about a third of the treated group showed some decrease in tumor size in the last week of treatment, suggesting some regression in this tumor model. Overall, these data suggest that neuroblastomas of the SK-N-AS subtype are somewhat more sensitive to resveratrol than those of the NGP subtype.

Bioavailability of the drug was found to be minimal, 2 to 10  $\mu\text{mol/L}$ , in the serum of nude mice 30 min after resveratrol was delivered by oral gavage. The concentration of resveratrol decreased thereafter, which is similar to results obtained by others (14). This is also similar to preliminary results from a recently initiated human phase I trial of resveratrol where serum levels of  $\sim 3 \mu\text{mol/L}$  were measured after an oral dosing of 70 mg/kg.<sup>6</sup> Furthermore, the drug does not accumulate in the tumor, liver, or serum during the 5-week treatment. Our experiments show that these relatively low levels are nonetheless sufficient to inhibit tumor growth and to cause some regression of SK-N-AS tumors. To enhance the local levels of resveratrol, a series of injections of the drug were given next to larger SK-N-AS tumors. Rapid tumor regression occurred during the injection regime, and after the last injection, most of the tumor had disappeared. Histology of these tumors showed

<sup>6</sup> Phase I repeat-dose clinical study of safety, pharmacokinetics and pharmacodynamics of resveratrol. Protocol UMCC Task 1 Pilot. 2006. Available from: <http://www-personal.umich.edu/~monk/protocols.html>.

dying tumor cells, whereas neighboring tissue generally remained unaffected. This suggests that, by using appropriate methods for delivery that bolster the local levels of resveratrol, the drug not only can prevent outgrowth of tumors but also can

have a strong antitumor action that leads to rapid regression of the tumor. In a xenograft model of gastric cancer, bolus injections of resveratrol next to the tumor caused an apoptotic index in the tumor of >50% (22).

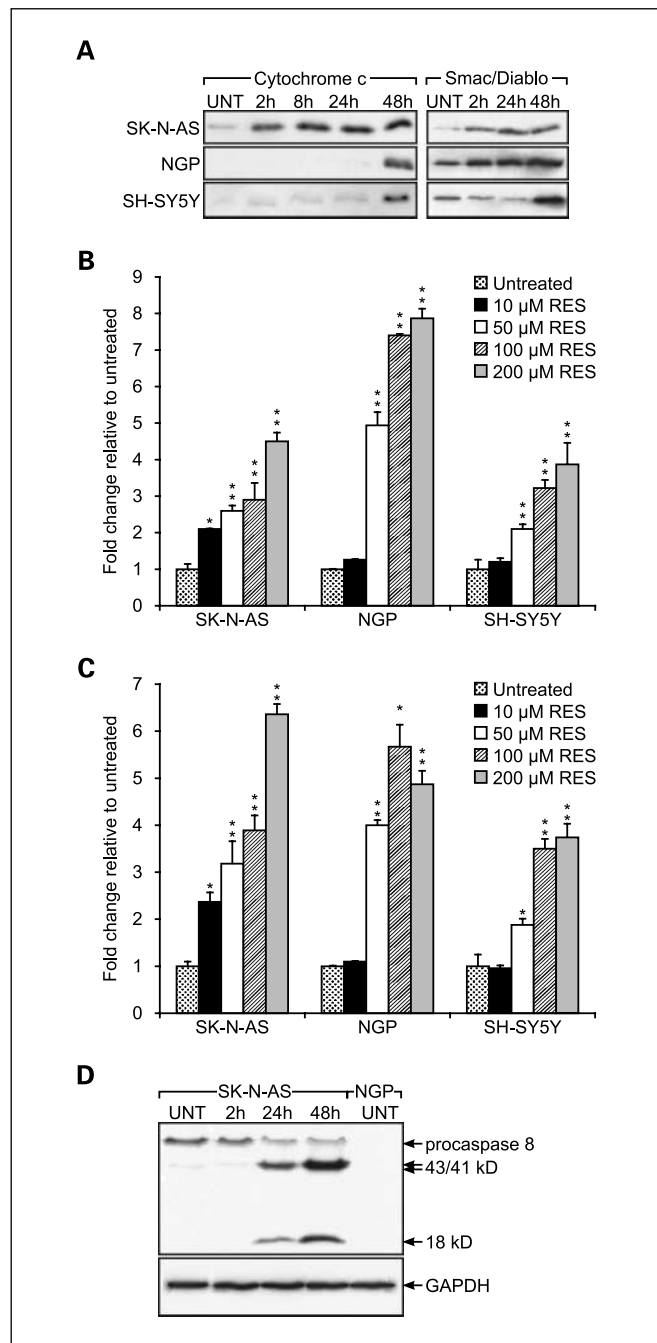
To understand the underlying mechanism of the antitumor activity of the drug, cell viability of neuroblastoma cell lines was studied *in vitro*. Loss of cell viability was observed in all cell lines studied with IC<sub>50</sub>s after 48 h of treatment that range between 70 and 120 μmol/L. Loss of cell viability can be accounted for in part by blocks in cell cycle progression caused by the drug, although these blocks occur at different stages of the cell cycle. The reason for these differences is currently unknown and may result from different levels of cell cycle regulatory proteins leading to differential responses to drug treatment. Moreover, resveratrol can inhibit DNA polymerase α and ribonucleotide reductase, which could cause the cells to arrest in S phase (23, 24). In addition, resveratrol-induced changes in levels and phosphorylation status of p21, p27, cyclins, cyclin-dependent kinases, and Rb have been reported (4).

The second major factor accounting for loss of cell viability is the induction of apoptosis in response to resveratrol treatment. All three cell lines display increased apoptosis after 24 to 48 h of drug treatment. Furthermore, after 8 to 10 days of treatment, all the cells are dead.

To further understand the mechanism of resveratrol-induced apoptosis, mitochondrial function was studied in response to drug treatment. Mitochondria are a central node in the intrinsic apoptotic pathway. In addition, mitochondria are important in their own right as the source of energy in the cell, especially considering the high metabolic rate of cancer cells. Membrane potential was used as a measure of mitochondrial function. Following resveratrol treatment, an early concentration-dependent decline in membrane potential was observed, indicating that resveratrol treatment of neuroblastoma cells leads to mitochondrial dysfunction.

To determine if resveratrol directly affects mitochondrial function and not indirectly via cytoplasmic signaling, isolated mitochondria were treated with the drug and mitochondrial membrane potential was measured. Similar to treatment of whole cells, treatment of isolated mitochondria with resveratrol resulted in loss of membrane potential in a concentration-dependent manner. These results suggest that one or multiple components of the mitochondria form direct targets for the drug. Because cancer cells are metabolically active due to their high mitotic index, they may be very sensitive to disruption of mitochondrial function as a result of resveratrol treatment. Others have described the F<sub>0</sub>F<sub>1</sub> ATPase/ATP synthase as a target for resveratrol action, although no direct binding studies have yet been done (12). In addition, resveratrol has been described to have effects on the mitochondrial respiratory chain (11) and the opening of the permeability transition pore (10). For these reasons, we are currently investigating potential mitochondrial targets.

To further delineate the mitochondrial pathway leading to apoptosis, cytochrome *c* release was measured in response to resveratrol treatment. Cytochrome *c* is released from mitochondria following apoptotic stimuli in some cell types (13) and may occur after loss of mitochondrial membrane potential. In SK-N-AS, NGP, and SH-SY5Y cells, resveratrol induces release of cytochrome *c*. However, treatment of isolated mitochondria with resveratrol does not lead to release of cytochrome *c*. This



**Fig. 5.** Resveratrol causes loss of cytochrome *c* and Smac/Diablo from the mitochondria and induces activation of caspase-9 and caspase-3. **A**, cytochrome *c* and Smac/Diablo were measured in the supernatants (cytoplasmic fractions) of SK-N-AS, NGP, and SH-SY5Y cells in a resveratrol time course. Fractions were analyzed by Western blot analysis for the presence of cytochrome *c* and Smac/Diablo. Each experiment was done in duplicate. UNT, untreated cells. Caspase-9 (**B**) and caspase-3 (**C**) activation was measured in SK-N-AS, NGP, and SH-SY5Y cells with the substrates Ac-LEHD-AFC and Ac-DEVD-AMC, respectively, in a resveratrol concentration course and plotted as relative fluorescence units after 48 h of drug treatment. Each experiment was done in triplicate. Caspase-8 (**D**) activation is induced by resveratrol in SK-N-AS cells but is absent in NGP cells. Caspase-8 levels were measured in whole-cell lysates by Western blot analysis. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) levels were measured as loading controls. \*\*,  $P < 0.0005$ ; \*,  $P < 0.05$ .

suggests that cytosolic proteins are required for resveratrol-induced cytochrome *c* release. Possible candidates are Bcl-2 family members, such as Bax, or BH3-only proteins, such as Bid, which have been shown to translocate to the mitochondria or activate proteins at the mitochondria following treatment with proapoptotic agents (reviewed in ref. 25). In addition to cytochrome *c* release, we also find release of Smac/Diablo from the mitochondria in response to resveratrol treatment.

Cytochrome *c* has been shown by others to form a complex with caspase-9 and APAF-1, thereby activating caspase-9 (13). Smac/Diablo has been shown to bind to inhibitor of apoptosis, thereby further activating caspase-9. This leads to further apoptotic signaling, particularly the activation of caspase-3. Both caspase-9 and caspase-3 were activated in SK-N-AS, NGP, and SH-SY5Y neuroblastoma cells in response to resveratrol. This suggests a pathway for resveratrol-induced apoptosis that originates in the mitochondria, leading to loss of membrane potential, release of cytochrome *c* and Smac/Diablo, and activation of caspase-9 and caspase-3, finally resulting in typical nuclear apoptotic morphology and cell death. Differences in the timing of the release of cytochrome *c* and Smac/Diablo and activation of caspase-9 and caspase-3 among the neuroblastoma cell lines may result from differences in the regulation of cytoplasmic components involved in these processes.

In conclusion, resveratrol strongly inhibits neuroblastoma tumor growth in animal models despite its low bioavailability in serum and tumor tissue following oral delivery. Because no obvious histopathologic signs of widespread apoptosis were observed under these conditions, it is more likely that inhibition of proliferation and/or angiogenesis underlies the inhibition of tumor growth at this dosage. A basic conundrum remains that the low bioavailability after oral dosing probably precludes tumor regression. Our mechanistic studies show that the drug induces apoptosis at considerably higher levels *in vitro*. These studies nevertheless suggest that if bioavailability can be increased either with respect to longevity or concentration of the drug *in vivo*, regression can be achieved. As proof of principle, resveratrol injected directly adjacent to the tumor resulted in rapid regression. Therefore, our current research is aimed at delivery routes, drug formulations, and drug derivatives to enhance the bioavailability of resveratrol. In addition, identifying resveratrol-binding proteins and the pathways in which they function may offer new targets for further drug intervention.

### Acknowledgments

We thank Joshua Harder for excellent technical assistance.

### References

- Gurney JG, Smith MA, Ross JA. Cancer among infants. In: Gloeckler Ries LA, editor. Cancer incidence and survival among children and adolescents: United States SEER Program, 1975-1995. Bethesda (MD): National Cancer Institute; 1999. p. 149-56.
- Ebb DH, Green DM, Shamberger RC, Tarbell NJ. Solid tumors of childhood. In: DeVita VT, Jr., Hellman S, Rosenberg SA, editors. Cancer: principles and practice of oncology. 6th ed. Philadelphia: Lippincott, Williams and Wilkins; 2001. p. 2169.
- Jang M, Cai L, Udeani GO, et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* 1997;275:218-20.
- Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, Takada Y. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. *Anticancer Res* 2004;24:2783-840.
- Liontas A, Yeager H. Curcumin and resveratrol induce apoptosis and nuclear translocation and activation of p53 in human neuroblastoma. *Anticancer Res* 2004;24:987-98.
- Chen Y, Tseng SH, Lai HS, Chen WJ. Resveratrol-induced cellular apoptosis and cell cycle arrest in neuroblastoma cells and antitumor effects on neuroblastoma in mice. *Surgery* 2004;136:57-66.
- Fulda S, Debatin KM. Resveratrol modulation of signal transduction in apoptosis and cell survival: a mini-review. *Cancer Detect Prev* 2006;30:217-23.
- Wang Z, Hsieh TC, Zhang Z, Ma Y, Wu JM. Identification and purification of resveratrol targeting proteins using immobilized resveratrol affinity chromatography. *Biochem Biophys Res Commun* 2004;323:743-9.
- Lin HY, Lansing L, Merillon JM, et al. Integrin  $\alpha_v\beta_3$  contains a receptor site for resveratrol. *FASEB J* 2006;20:1742-4.
- Tinohofer I, Bernhard D, Senfter M, et al. Resveratrol, a tumor-suppressive compound from grapes, induces apoptosis via a novel mitochondrial pathway controlled by bcl-2. *FASEB J* 2001;15:1613-5.
- Zini R, Morin C, Bertelli A, Bertelli AA, Tillement JP. Effects of resveratrol on the rat brain respiratory chain. *Drugs Exp Clin Res* 1999;25:87-97.
- Zheng J, Ramirez VD. Inhibition of mitochondrial proton F0F1-ATPase/ATP synthase by polyphenolic phytochemicals. *Br J Pharmacol* 2000;130:1115-23.
- Jiang X, Wang X. Cytochrome C-mediated apoptosis. *Annu Rev Biochem* 2004;73:87-106.
- Asensi M, Medina I, Ortega A, et al. Inhibition of cancer growth by resveratrol is related to its low bioavailability. *Free Radic Biol Med* 2002;33:387-98.
- Sareen D, van Ginkel PR, Takach JC, et al. Mitochondria as the primary target of resveratrol-induced apoptosis in human retinoblastoma cells. *Invest Ophthalmol Vis Sci* 2006;47:3708-16.
- Lastowska M, Cullinane C, Variend S, et al. Comprehensive genetic and histopathologic study reveals three types of neuroblastoma tumors. *J Clin Oncol* 2001;19:3080-90.
- Larrosa M, Tomás-Barberán FA, Espín JC. Grape polyphenol resveratrol and the related molecule 4-hydroxystilbene induce growth inhibition, apoptosis, S-phase arrest, and upregulation of cyclins A, E, and B1 in human SK-mel-28 melanoma cells. *J Agric Food Chem* 2003;51:4576-84.
- Ahmad N, Adhami VM, Afaq F, Feyes DK, Mukhtar H. Resveratrol causes WAF-1/p21-mediated G(1)-phase arrest of cell cycle and induction of apoptosis in human epidermoid carcinoma A431 cells. *Clin Cancer Res* 2001;7:1466-73.
- Lucken-Ardjomande S, Martinou JC. Regulation of Bcl-2 proteins and of the permeability of the outer mitochondrial membrane. *C R Biol* 2005;328:616-31.
- Anto RJ, Mukhopadhyay A, Denning K, Aggarwal BB. Curcumin (diferuloylmethane) induces apoptosis through activation of caspase-8, BID cleavage and cytochrome *c* release: its suppression by ectopic expression of bcl-2 and bcl-xl. *Carcinogenesis* 2002;23:143-50.
- Fulda S, Poremba C, Berwanger B, et al. Loss of caspase-8 expression does not correlate with MYCN amplification, aggressive disease, or prognosis in neuroblastoma. *Cancer Res* 2006;66:10016-23.
- Zhou HB, Chen JJ, Wang WX, Cai JT, Du Q. Anticancer activity of resveratrol on implanted human primary gastric carcinoma cells in nude mice. *World J Gastroenterol* 2005;11:280-4.
- Locatelli GA, Savio M, Forti L, et al. Inhibition of mammalian DNA polymerases by resveratrol: mechanism and structural determinants. *Biochem J* 2005;389:259-68.
- Fontecave M, Lepoivre M, Elleingand E, Gerez C, Guittet O. Resveratrol, a remarkable inhibitor of ribonucleotide reductase. *FEBS Lett* 1998;421:277-9.
- Scorrano L, Korsmeyer SJ. Mechanisms of cytochrome *c* release by proapoptotic BCL-2 family members. *Biochem Biophys Res Commun* 2003;304:437-44.