

# Brain-derived Neurotrophic Factor Protects Neuroblastoma Cells from Vinblastine Toxicity

Stefania Scala, Katja Wosikowski, Paraskevi Giannakakou, Paola Valle, June L. Biedler, Barbara A. Spengler, Enrico Lucarelli, Susan E. Bates, and Carol J. Thiele<sup>1</sup>

Medicine Branch, Division of Cancer Treatment [S. S., K. W., P. G., S. E. B.], and Cell and Molecular Biology Section, Pediatric Branch [P. V., E. L., C. J. T.], National Cancer Institute, Bethesda, Maryland 20892, and Laboratory of Cell and Biochemical Genetics, Memorial Sloan Kettering Cancer Center, New York, New York 10021 [J. L. B., B. A. S.]

## ABSTRACT

Brain-derived neurotrophic factor (BDNF) and its receptors are necessary for the survival and development of many neuronal cells. Because BDNF and TrkB are expressed in many poor-prognosis neuroblastoma (NB) tumors, we evaluated the role of BDNF in affecting sensitivity to chemotherapeutic agents. We investigated the effects of activation of the BDNF-TrkB signal transduction pathway in two NB cell lines, 15N and SY5Y. 15N cells lack the high-affinity receptor p145TrkB and express BDNF; 15N cells were used along with 15N-TrkB cells, a subline transfected with a TrkB expression vector. In cytotoxicity assays, 15N-TrkB cells were consistently 1.4–2-fold more resistant to vinblastine than 15N cells. Drug accumulation assays showed a 50% reduction in [<sup>3</sup>H]vinblastine accumulation in 15N-TrkB cells compared with control 15N cells. Addition of 30 ng/ml BDNF resulted in a reduction to 46% of control in 15N cells and a reduction to 28% of control in 15N-TrkB cells. SY5Y cells were chosen as a second model because they lack both endogenous BDNF and TrkB expression. p145TrkB expression is induced by 1 nM retinoic acid. Vinblastine accumulation was not significantly affected by 1 nM retinoic acid in SY5Y cells. Addition of 30 ng/ml BDNF decreased [<sup>3</sup>H]vinblastine accumulation to 58% of control in SY5Y cells and decreased [<sup>3</sup>H]vinblastine accumulation to 62% of control in TrkB-expressing SY5Y cells. Although an increase in BDNF expression is seen in multidrug-resistant sublines of SY5Y and BE(2)-C NB cells, the protective effect of BDNF in vinblastine toxicity may be unrelated to *mdr-1*, because the activity of other agents transported by P-glycoprotein was not affected. There was no increase in *mdr-1* expression in 1 nM RA SY5Y cells and 15N-TrkB cells, as assessed by Northern blot analysis. In addition to the effects of BDNF on vinblastine cytotoxicity and accumulation, there was an inhibition in the ability of vinblastine to depolymerize tubulin in BDNF-treated cells. Thus, BDNF and TrkB may partially rescue NB cells from vinblastine toxicity and thereby may contribute to a more chemoresistant phenotype.

## INTRODUCTION

NB<sup>2</sup> is a neural crest-derived tumor of early childhood (1). Stratification of tumors into good and poor prognostic categories is based on age, tumor location, DNA ploidy, *N-myc* amplification, and expression of TrkA, the high-affinity NGF receptor (1). Good-prognosis NB tumors express relatively high levels of TrkA, whereas cell lines and tumors from patients with a poor prognosis frequently express BDNF and, variably, its receptor TrkB (1, 2).

Neurotrophins play an essential role in the growth, survival, and differentiation of neurons in both the peripheral and central nervous systems. NGF (3) is the best characterized member of the neurotrophin family, which includes BDNF, NT-3, and NT-4/5. The activity of

neurotrophins is mediated by the Trk family of tyrosine kinase receptors. TrkA is the receptor for NGF but also binds NT-3 and NT-4/5. TrkB is the BDNF receptor but also binds NT-3 and NT-4/5. TrkC is the receptor for NT-3. In addition, all neurotrophins bind to a low-affinity receptor, p75NGFR, with similar affinity. The biological effects of activation of the TrkA receptor include cell growth arrest, neurite induction, and neuronal cell survival (4, 5). In contrast with the NGF effect, BDNF does not inhibit NB cell proliferation but increases cell survival and stimulates neurite extension and cellular invasion through extracellular matrix protein (6, 7). Because poor-prognosis NB tumors preferentially express BDNF-TrkB (7), it is possible that differential expression of neurotrophins and activation of their respective signal transduction pathways directly affects the biological behavior of the tumor cell. How BDNF promotes cell survival is not fully understood, but investigators working in several model systems have confirmed a protective effect of BDNF. Neurotrophins such as BDNF and NT-4/5 promote survival and differentiation of striatal neurons *in vitro* (8). Acheson *et al.* (9) described a BDNF autocrine loop able to prevent cell death in dorsal root ganglion neurons. They showed that treatment with antisense against BDNF reduced the neuronal survival by 35%, and that exogenous addition of BDNF or NT-3, but not other neurotrophins, could rescue them (9). Also, BDNF has been described as a survival factor with a role in protection against metabolic and excitotoxic insults in central nervous system neurons (10). In SY5Y and KCN NB cells, BDNF increases cell survival (6, 7). Thus, several biologically important events involving neurotrophins are involved in the response of neuronal cells to injury and may provide a survival advantage in neuronal cells (5, 11–14).

Although most patients with poor-prognosis NB are initially sensitive to aggressive chemotherapeutic treatment, most relapse and become refractory to the therapy (1). *In vitro*, the most common cause of chemoresistance in NB is overexpression of Pgp, the product of the *mdr-1* gene, (15, 16). However, conflicting reports have appeared regarding the role of Pgp in conferring clinical drug resistance or poor prognosis (17–19). Recent evidence also indicates that MRP expression in neuroblastomas is associated with a poor prognosis (20).

Because BDNF is a survival factor and exerts a protective role on neuronal cells, we reasoned that the constitutive expression of BDNF and TrkB by poor-prognosis NB tumor cells may contribute to a chemoresistant phenotype in NB. Thus, the effect of BDNF on chemosensitivity in unselected NB cell lines was analyzed.

## MATERIALS AND METHODS

**Cell Cultures.** SH-SY5Y (SY5Y), BE(2)-C, and La1–15N human NB cells were cultured in RPMI medium containing 10% fetal bovine serum, 100 IU penicillin/ml, and 50 mg/ml streptomycin. To obtain a subline transfected with the TrkB receptor, La1–15N cells were transfected with 10  $\mu$ g pIRVNeoCMV-TrkB and cDNA using electroporation. Geneticin-resistant cells expressing p145TrkB (15N-TrkB) were isolated and characterized (21). To obtain a suitable control, an identical population of La1–15N cells were transfected with 10  $\mu$ g control plasmid pIRV-NeoCMV (15N), containing a geneticin resistance gene, using electroporation. Transfected cells were maintained in 300  $\mu$ g/ml geneticin (21).

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<sup>1</sup> To whom requests for reprints should be addressed, at Cell and Molecular Biology Section, Pediatric Branch, National Cancer Institute, Bethesda, MD 20892. Phone: (301) 496-1543; Fax: (301) 402-0575; E-mail: thielec@pbmac.nci.nih.gov.

<sup>2</sup> The abbreviations used are: NB, neuroblastoma; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; LNGFR, low affinity NGF receptor; Pgp, P-glycoprotein; *mdr*, multidrug resistance; MRP, multidrug resistance-associated protein; RA, retinoic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NT, neurotrophin; Trk, tropomyosin receptor kinase.

SY5Y/ACT(1) cells were selected from parental SY5Y cells by stepwise increases in the concentration of actinomycin D and were maintained in medium containing 1 mg/ml of the drug. SY5Y/VCR(10) cells were similarly selected with vincristine and are carried in 10 mg/ml vincristine. BE(2)-C/ACT(1), BE(2)-C/ADR(5), and BE(2)-C/VCR(10) cells were selected by stepwise increases in drug concentration from BE(2)-C cells and were carried in 1 mg/ml actinomycin D, 5 mg/ml doxorubicin, and 10 mg/ml vincristine, respectively (22).

**Drug Accumulation.** Drug accumulation studies were performed as reported previously (23). Briefly,  $3.5 \times 10^5$  cells were plated into each well of a six-well plate. 15N control or 15N-TrkB cells were incubated for 5 days following the day of plating. SY5Y cells were treated 24 h later with 1 nM RA and incubated an additional 5 days. Cells were rinsed and incubated with assay medium prior to initiation of the drug accumulation assay, which was performed using 14 nM [ $^3\text{H}$ ]vinblastine ( $2 \times 10^6$  dpm/well) in the absence or presence of BDNF.

**Cytotoxicity Assay.** Cytotoxicity studies on 15N and 15N-TrkB cells were performed in 96-well plates. Cells were plated at 2000 cells/well and treated the day after plating with actinomycin D, vinblastine, or paclitaxel. After 5 days in the drugs, the cells were fixed in 10% trichloroacetic acid and stained with 0.4% sulphorhodamine B in 1% acetic acid, as described previously (24). The bound dye was solubilized in 200  $\mu\text{l}$  of 10 mM unbuffered Tris solution. The absorbance was determined at a wavelength of 540 nm in an ELISA microplate reader (Bio-Rad) in quadruplicate. Untreated control wells were assigned a value of 100%. The  $\text{IC}_{50}$  is defined as the dose of the drug required to inhibit cells to 50% of the control value. For cytotoxicity experiments with SY5Y cells,  $5 \times 10^4$  cells/well were plated in six-well dishes and treated 24 h later with 1 nM RA for 5 days. Media were removed, and the cells were incubated with vinblastine in the presence or absence of 30 ng/ml BDNF for 4 h. Following trypsinization, the cells were counted and seeded in 96-well plates (2000 cells/well). After the cells had attached overnight, one plate (day 0) was harvested, whereas the remaining plate (day 5) was harvested 5 days later, as described above. The values obtained on day 5 ( $n = 8$ ) were divided by those from day 0 ( $n = 8$ ) to correct for differences in cell numbers when replated and were expressed as percentages of control  $\pm$  SD. The control represents the results from cells at day 5 not treated with vinblastine.

**Northern Blot Analysis.** RNA isolation and hybridization were performed as described previously (25). Thirty  $\mu\text{g}$  total RNA for BDNF and GAPDH and 8  $\mu\text{g}$  for *mdr-1* were electrophoresed in 1% agarose-6% formaldehyde gels. Gels were stained with 2 mg/ml ethidium bromide to allow inspection of the quantity and quality of RNA. For *mdr-1*, hybridization was performed as described previously with a  $^{32}\text{P}$ -labeled synthetic riboprobe corresponding to nucleotides 1176–2555 of the *mdr-1* gene (26). For BDNF and GAPDH, hybridization was performed with  $^{32}\text{P}$ -labeled insert DNA isolated from a plasmid containing BDNF (27) and GAPDH.

**Tubulin Polymerization Assay.** To measure the degree of tubulin polymerization, we used a modification of the method originally described by Minotti *et al.* (28). SY5Y cells treated for 5 days with 1 nM RA were plated in 24-well tissue culture plates. The following day, the cells were treated with 150 ng/ml vinblastine for 3 h in the presence or absence of 30 ng/ml BDNF. Cells were then washed twice in PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  and lysed in 100  $\mu\text{l}$  hypotonic buffer [20 mM Tris-HCl (pH 6.8), 1 mM  $\text{MgCl}_2$ , 2 mM EGTA, 0.5% NP40, 2 mM phenylmethylsulfonyl fluoride, 200 units/ml aprotinin, 100  $\mu\text{g}/\text{ml}$  soybean trypsin inhibitor, 5.0 mM  $\epsilon$ -aminocaproic acid, and 1.0 mM benzamide with or without 4  $\mu\text{g}/\text{ml}$  paclitaxel at 37°C in the dark. Cell lysates were centrifuged at 14,000 rpm for 10 min at room temperature. Supernatants containing soluble (cytosolic) tubulin were carefully separated from pellets containing polymerized (cytoskeleton) tubulin. The pellets were resuspended in 200  $\mu\text{l}$  lysis buffer.

**Protein Electrophoresis and Immunodetection.** Protein cell lysates were analyzed by SDS-PAGE and transferred to Immobilon membranes, which were blocked in 10% nonfat dry milk and 0.5% Tween 20 in PBS for 1 h, and then incubated with primary anti- $\alpha$ -tubulin antibody (Sigma Chemical Co.) diluted 1:1000. Membranes were washed three times with 0.5% Tween 20 in PBS (wash buffer) before incubation with the secondary horseradish peroxidase-conjugated antibody diluted 1:2000 in the blocking solution. The substrate was visualized by enhanced chemiluminescence reaction (Amersham).

## RESULTS

**NB Cell Models.** To test the effects of BDNF and activation of the TrkB signal transduction pathways on drug sensitivity, we used two unselected NB cell lines, shown in the schematic in Fig. 1. In the 15N model system, cells produce BDNF but do not express TrkB mRNA by Northern analysis or p145TrkB by Western analysis (21). Stable TrkB-expressing 15N (15N-TrkB) cells have been generated by transfection with pIRVneoCMVratTrkB cDNA, whereas 15N cells transfected with the empty vector served as a control in every experiment (21). In the SY5Y model, the cells do not express readily detectable levels of TrkB or BDNF by Northern analysis (21). Treatment with nanomolar concentrations of RA induces p145TrkB and its kinase-inactive form, p95TrkB; subsequently, the addition of BDNF causes receptor dimerization, TrkB autophosphorylation, and signal transduction (21). Previous studies have also shown that nanomolar RA does not inhibit cell proliferation (6).

**Effect of RA and BDNF on *mdr-1* Pgp Expression.** As reported previously, treatment of NB cells with higher doses of RA (1–5  $\mu\text{M}$ ) increases the expression of Pgp, the product of the *mdr-1* gene (29). To examine changes in *mdr-1* expression following RA treatment, the *mdr-1* mRNA levels were evaluated in both cell models under all conditions used. As shown in Figure 2A, there was a 2-fold increase in *mdr-1* mRNA levels in SY5Y cells treated with 1 nM RA, as determined by densitometric analysis of *mdr-1* mRNA levels normalized to GAPDH levels. There were, however, 14- and 16-fold increases in *mdr-1* expression after treatment with higher doses of RA, 1  $\mu\text{M}$  and 5  $\mu\text{M}$ , respectively. The addition of BDNF to TrkB-expressing SY5Y cells did not significantly alter *mdr-1* mRNA levels (Fig. 2A). Furthermore, there was no difference in the *mdr-1* level in the 15N control and TrkB-transfected cell line (Fig. 2B). In addition, 15N and 15N-TrkB cells expressed equivalent levels of low-affinity NGF receptor LNGFR mRNA (Fig. 2C).

**Effect of BDNF on Drug Sensitivity in SY5Y and 15N NB Cells.** Because the *mdr-1* level is slightly affected, we asked whether BDNF or TrkB could modulate the sensitivity to chemotherapeutic agents. To

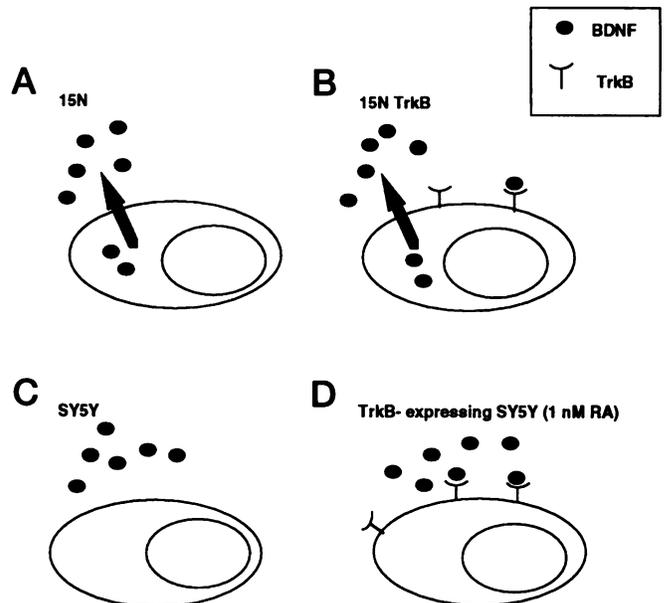


Fig. 1. NB cell models. A, 15N cells express BDNF but not TrkB receptor. B, 15N-TrkB cells express TrkB receptor; addition of BDNF results in a functional BDNF-TrkB pathway. C, SY5Y cells do not express BDNF or TrkB. Exogenous BDNF has no TrkB receptor to bind to. D, TrkB-expressing SY5Y cells. Five days of treatment with 1 nM RA induced TrkB receptor in SY5Y cells. Exogenous BDNF binds TrkB receptor (21).

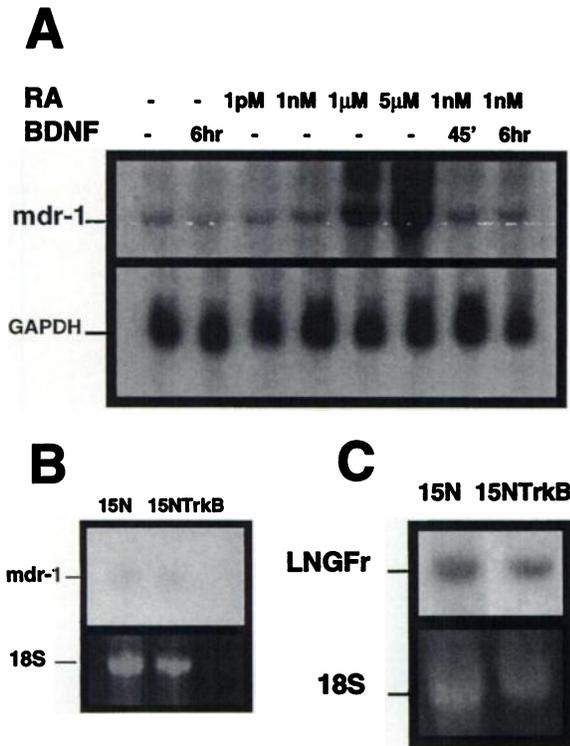


Fig. 2. Effect of RA acid treatment on *mdr-1*/Pgp expression. A, SY5Y cells treated for 5 days with different concentrations of RA (1 pM, 1 nM, and 1 and 5 μM) and 1 nM RA-treated cells were treated with 30 ng/ml BDNF for 45 min or 6 h. Northern blot analysis was performed as described in "Materials and Methods." Briefly, 8 μg total RNA were separated in a 1% agarose-6% formaldehyde gel, transferred to nylon filters, and hybridized with riboprobe detecting *mdr-1*. Bottom panel, GAPDH hybridization is depicted. B, Northern analysis of *mdr-1* mRNA levels in 20 μg 15N and 15N-TrkB total RNA. Bottom panel, ethidium-stained 18S RNA. C, Northern analysis of NGFR mRNA in 20 μg 15N and 15N-TrkB total RNA. Bottom panel, ethidium-stained 18S RNA.

study the effect of cytotoxic drugs on cells with an activated TrkB signal transduction pathway, 15N and 15N-TrkB cells that constitutively express BDNF were exposed to different concentrations of vinblastine, actinomycin D, and paclitaxel. Cytotoxicity curves were generated using serial dilutions of vinblastine, actinomycin D, and paclitaxel from 3 μg/ml to 0.03 ng/ml. As shown in Fig. 3, in 15N-TrkB cells the vinblastine IC<sub>50</sub> was 1.2 ng/ml, compared with the parental 15N IC<sub>50</sub> of 0.6 ng/ml, whereas in both 15N and 15N-TrkB cells the IC<sub>50</sub> was 0.9 ng/ml for actinomycin D and 0.8 ng/ml for paclitaxel. 15N-TrkB cells were consistently 1.4–2-fold more resist-

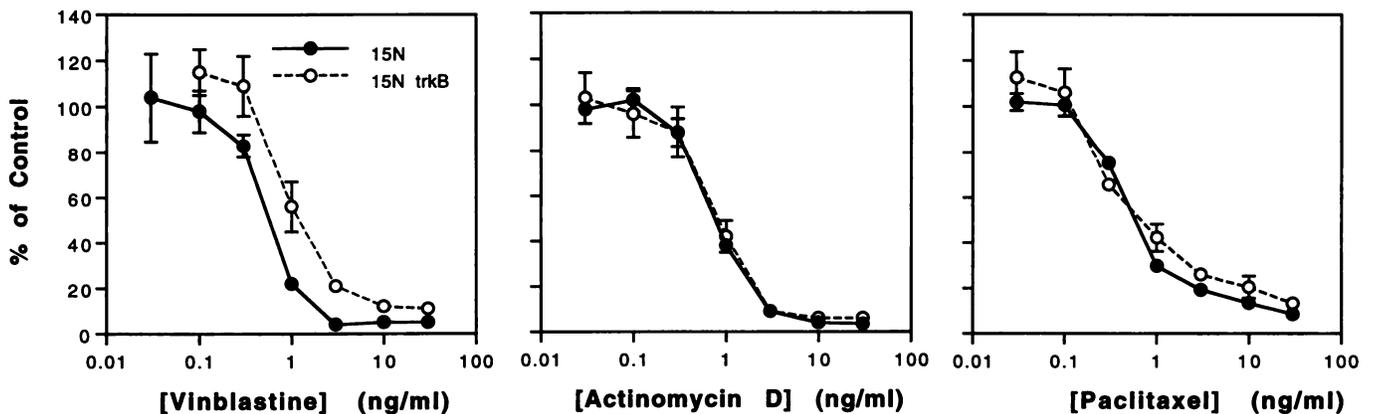


Fig. 3. Effect of TrkB expression on vinblastine, actinomycin D, and paclitaxel sensitivity in 15N-TrkB cells. 15N and the TrkB-transfected 15N-TrkB cells were seeded (2000 cells/well) in 96-well plates. Twenty-four h later, cells were treated with vinblastine, actinomycin D, and paclitaxel for 5 days and harvested as described in "Materials and Methods." These results were obtained in three different experiments ( $P < 0.002$ ).

ant to vinblastine yet were unchanged in their sensitivity to actinomycin D and paclitaxel.

To study the effect of cytotoxics on cells expressing TrkB and BDNF, SY5Y cells treated with 1 nM RA were used. TrkB-expressing SY5Y cells exposed to either vinblastine alone or vinblastine plus BDNF (30 ng/ml) showed a 1.6-fold increase in resistance to vinblastine from an IC<sub>50</sub> of 0.45 to 0.74 μg/ml in the presence of BDNF (Fig. 4). The effect of BDNF was reproducible in three experiments performed. The modification in vinblastine sensitivity, albeit modest, indicates that in both model systems BDNF and TrkB expression may increase vinblastine resistance or cell survival.

**Effect of BDNF on Drug Accumulation.** To determine whether the difference in drug sensitivity could reflect altered drug accumulation, cells were incubated with [<sup>3</sup>H]vinblastine in the absence or presence of BDNF, and intracellular drug accumulation was measured after 45 min. As shown in Fig. 5, vinblastine accumulation in 15N-TrkB cells was 50% of the levels in control 15N cells. The addition of BDNF reduced vinblastine accumulation to 46% of control in 15N cells and to 28% of control in the 15N-TrkB cells. This indicates that BDNF stimulates a decrease in vinblastine accumulation in 15N cells.

Drug accumulation was also evaluated in the SY5Y cell model system. Control or TrkB-expressing SY5Y cells (RA-SY5Y) were incubated for 45 min with [<sup>3</sup>H]vinblastine in the absence or presence of BDNF. BDNF treatment resulted in a decrease in the drug to 58% compared with the control SY5Y cells (Fig. 5). The TrkB-expressing SY5Y (RA-SY5Y) cells did not show a significant decrease in drug accumulation in the absence of BDNF compared with untreated SY5Y cells. However, the addition of BDNF to TrkB-SY5Y (RA and BDNF) resulted in a decrease in drug accumulation to 62% compared with untreated SY5Y cells without TrkB expression. The drug accumulation results in control 15N and SY5Y cells suggest that the action of BDNF on drug accumulation may have a component that is independent from the expression of p145TrkB, because SY5Y and 15N cells do not express readily detectable TrkB, as measured by Northern and Western analyses. This reduction in accumulation was highly reproducible and observed in four separate experiments in each model system.

**Effect of BDNF on Tubulin in NB Cells.** To further define the effect of BDNF on vinblastine sensitivity in NB cells, we evaluated the effect of BDNF on tubulin, the intracellular target of vinblastine. In a normal cell, tubulin is present in a dynamic equilibrium between polymerized and unpolymerized forms. Tubulin assays are used to identify the ratio of polymerized:unpolymerized tubulin (28). Typically, vinblastine causes tubulin depolymerization, resulting in a shift

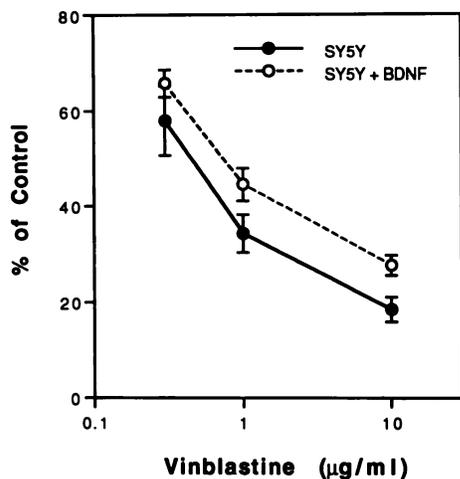


Fig. 4. BDNF effect on vinblastine sensitivity in TrkB-expressing SY5Y cells. SY5Y cells were seeded (50000 cells/ml) in 24-well plates and treated with 1 nM RA for 5 days. Then the media were removed, and the cells were treated with vinblastine alone or vinblastine and 30 ng/ml BDNF for 4 h. The cells were then counted and plated in 96-well plates, and harvesting was performed as described. These results are the mean of three separate experiments ( $P < 0.002$ ). Bars, SD.

in tubulin from the polymerized state found in the cell pellet to the unpolymerized state found in the cell supernatant. Fig. 6 demonstrates a decrease in polymerized tubulin in cell lysates harvested in low-salt buffer in vinblastine-treated TrkB-SY5Y cells. BDNF treatment alone caused a decrease in tubulin found in the polymerized state, although not to the extent seen in cells treated with vinblastine. However, when TrkB-SY5Y cells were treated with vinblastine in the presence of BDNF, there was a decrease in the extent of vinblastine-induced tubulin depolymerization.

The low level of polymerized tubulin found in SY5Y cells made it difficult to evaluate the small changes induced by vinblastine and the effect of BDNF. For this reason, additional experiments were performed in which cells were harvested in the presence of paclitaxel, a drug with an effect on the cytoskeleton that is associated with an increase in tubulin polymerization. When paclitaxel is added to TrkB-SY5Y cells, there is an almost equal distribution of tubulin in the supernatant and pellet fractions. Under these conditions, BDNF stimulates a slight decrease in tubulin polymerization, whereas vinblastine causes a dramatic shift in tubulin to the depolymerized state (supernatant). However, when cells are treated with both vinblastine and BDNF, there is a marked reduction in the depolymerization induced by vinblastine.

**BDNF Expression in Drug-resistant NB Cells.** Because BDNF protects NB cells from vinblastine cytotoxicity, we asked whether there was altered expression of BDNF in NB cells resistant to vincristine (a *Vinca* alkaloid related to vinblastine). Northern blot analysis was performed in two NB cell lines and their multidrug-resistant sublines. As shown in Fig. 7, left panel, increased expression of BDNF mRNA was found in the multidrug-resistant sublines of both SY5Y and BE(2)-C cells, compared with the level detected in the parental cells. A 3-fold increase in BDNF mRNA was detected in SY5Y-VCR cells, whereas BDNF mRNA levels in SY5Y-ACT cells were not significantly altered. Relative to parental cells, BDNF mRNA levels were increased 18-fold in vincristine-resistant BE(2)-C sublines. Additionally, there were 20- and 16-fold increases in BDNF levels in actinomycin D- and Adriamycin-resistant cell lines, respectively. As shown in Fig. 7, right panel, BE(2)-C cells analyzed at two steps of Adriamycin or colchicine selection demonstrated an increase in BDNF mRNA, indicating a relationship between increased drug resistance and BDNF mRNA levels. Northern analysis indicated there was no change in the constitutive expression of TrkB mRNA in these

cell lines (data not shown). Thus, not only is BDNF expression increased in vincristine-resistant NB cells, but it is also increased in some cell lines resistant to other drugs.

**DISCUSSION**

The results presented here show that BDNF increases cellular resistance to vinblastine in 15N and SY5Y unselected NB cell lines. In two different NB cell models expressing the TrkB receptor, 15N-

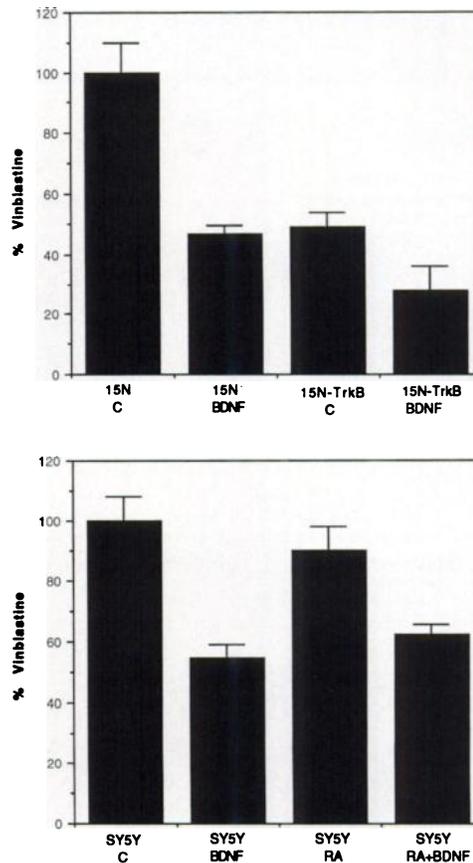


Fig. 5. Effect of BDNF on vinblastine accumulation. Top panel, 15N and 15N-TrkB cells were plated (350,000 cells/ml) in six-well plates. [<sup>3</sup>H]Vinblastine accumulation was performed with and without 30 ng/ml BDNF as described in "Materials and Methods." Bottom panel, SY5Y cells were treated with 1 nM RA for 5 days prior to plating. These results are the mean of four separate experiments. Bars, SD.

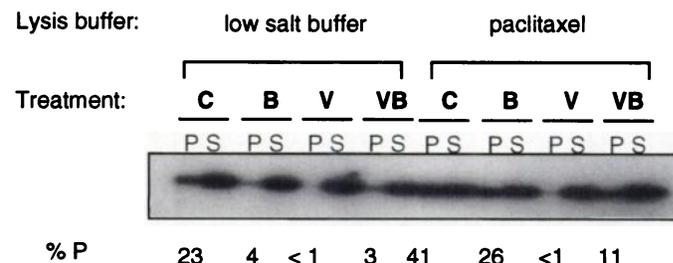
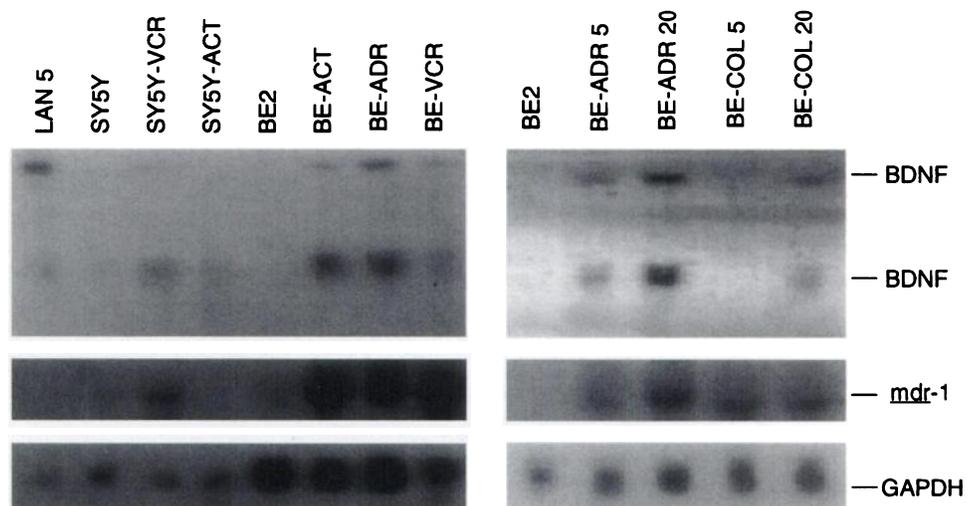


Fig. 6. Effect of BDNF on tubulin distribution in TrkB-expressing SY5Y cells. SY5Y cells were treated for 5 days with 1 nM RA. The cells were then seeded in 24-well plates (200,000 cells/ml); the day after, the cells were treated with 150 ng/ml vinblastine, 30 ng/ml BDNF, or vinblastine plus BDNF for 3 h. Cells were then harvested in the absence (low salt buffer) or presence of paclitaxel (4 mg/ml) buffer, divided in soluble and insoluble fractions, as described in "Materials and Methods," and Western blot analysis for tubulin was performed. C, untreated cells; B, 30 ng/ml BDNF; V, 150 ng/ml vinblastine; VB, 50 ng/ml vinblastine and 30 ng/ml BDNF; P, pellet; S, supernatant. Bottom panel, densitometric quantitation expressed as percentage of the polymerized (P) tubulin. These results are the mean of three different experiments.

Fig. 7. Expression of BDNF and *mdr-1*/Pgp in multidrug-resistant human NB cells: Northern blot analysis in human NB parental and multidrug-resistant cells. Twenty-five  $\mu$ g total RNA for BDNF, GAPDH, and *mdr-1* were separated in a 1% agarose-6% formaldehyde gel, transferred to nylon filters, and hybridized with a riboprobe detecting BDNF, *mdr-1*, and GAPDH, as described in "Materials and Methods."



TrkB and SY5Y cells treated with RA, we report increased vinblastine resistance after treatment with BDNF. This was demonstrated by an almost 2-fold increase in the  $IC_{50}$  for vinblastine and was accompanied by a reduction in vinblastine accumulation. In contrast, BDNF did not significantly modify the sensitivity of SY5Y and 15N-TrkB cells to other drugs involved in the mdr phenotype, such as actinomycin D and paclitaxel. Because the alteration in chemosensitivity was specific to vinblastine and did not affect actinomycin D or paclitaxel, it is unlikely that the small changes in *mdr-1* expression stimulated by RA or BDNF were solely responsible for the effects of BDNF on vinblastine sensitivity. Furthermore, treatment with 30 ng/ml BDNF in TrkB-expressing SY5Y and 15N-TrkB cells did not affect rhodamine 123 efflux, a known substrate for Pgp (data not shown; Ref. 30). The finding that BDNF levels are increased in vincristine-resistant NB cell lines is consistent with the data that BDNF affects vinblastine cytotoxicity. Under the conditions studied, the BDNF effect on vinblastine sensitivity is apparently independent of Pgp in these model systems. However, it is interesting that cells resistant to other drugs and overexpressing Pgp also have increased BDNF levels. It is possible that BDNF may have a broader role in drug resistance in NB that was not detected in the model systems and conditions studied due to the sensitivities of the assays. Although increased levels of another drug resistance gene, *MRP*, have been detected in NBs with a poor prognosis (20), we did not detect any significant alteration in *MRP* mRNA levels in the SY5Y cells in the presence or absence of RA or BDNF.<sup>3</sup>

There are several potential mechanisms by which BDNF could act. In classic neurobiological studies, Johnson (12) showed that a single injection of vinblastine administered to neonatal rats produced massive cell death in the superior cervical ganglia, yet these cytotoxic effects could be prevented by the concomitant administration of NGF. In these experiments, it was postulated that the binding of neurotrophins and their receptors to cytoskeletal elements as part of the retrograde transport system in neurons inhibited the effects of vinblastine. Retrograde transport is one mechanism proposed to account for the ability of neurotrophic factors in a target organ to ensure innervation, survival, and function of distant neuronal cell bodies (31–33). Both the low-affinity neurotrophin receptor p75NGFR (4, 34) and the high-affinity BDNF receptor p145TrkB (35, 36) have been shown to bind to cytoskeletal elements and to be transported in a retrograde fashion from nerve termini to the cell body. Because the

BDNF effect described here is also in SY5Y and 15N cells that do not express readily detectable levels of TrkB (6, 7, 21, 37), it is possible that a component of the effect that we measured is mediated via the low-affinity p75NGFR. SY5Y cells (38) and both of the 15N cell lines (Fig. 2C) express p75NGFR. Signal transduction via the low-affinity neurotrophin receptor alone has not been well studied, but recent evidence indicates that neurotrophins binding p75NGFR in the absence of Trk activate sphingomyelin hydrolysis and stimulate ceramide-mediated signal transduction (39, 40). Current studies will evaluate the role of this signal transduction path in mediating vinblastine resistance. We cannot exclude, however, that both the SY5Y and the 15N cell lines express levels of TrkB below the limits of detection in biochemical assays but are sensitive to the biological effects of neurotrophins. Increased expression of the early response gene *fos* has been detected in BDNF-treated SY5Y cells (41)<sup>4</sup> and rat cortical glial cells, which also did not express readily detectable levels of the high-affinity receptors (42).

A second possible mechanism for the specific vinblastine resistance in our system is posttranslational modification of tubulin by BDNF. The target for vinblastine and other *Vinca* alkaloids is tubulin, which is the basal unit of microtubules. Mutations or altered expression of tubulin may account for paclitaxel and vinblastine resistance in human ovarian carcinoma cells and murine J774.2 cells (43, 44).<sup>5</sup> Because posttranslational modifications (tyrosination, acetylation, glutamylation, and phosphorylation) of tubulin have been described elsewhere, it is possible that BDNF could modify tubulin, resulting in decreased tubulin binding (45). A decrease in total vinblastine accumulation could result, because less target is available to bind. An alternative possibility is that the decrease in tubulin polymerization observed following BDNF treatment is due to the lower vinblastine accumulation stimulated by BDNF rather than a direct effect of BDNF on tubulin.

A third possible mechanism for the effect of BDNF in promoting resistance is that of a survival factor (8, 14). Previous results have suggested that the BDNF-TrkB pathway plays a role in cell death in sensory neurons (9), and that BDNF is a survival factor for cerebellum granule neurons in culture (11). Barde (46) found that the total number of degenerating neurons in developing dorsal root ganglia could be significantly reduced by administering BDNF. In our system, the BDNF pathway may protect NB cells by conferring increased survival in the presence of vinblastine; however, if this were the only mech-

<sup>3</sup> Unpublished data.

<sup>4</sup> Unpublished results.

<sup>5</sup> P. Giannakou, unpublished results.

anism, one would have expected an alteration in response to the other drugs as well.

Our results indicate that BDNF treatment reproducibly decreases vinblastine chemosensitivity in unselected NB cells. The clinical significance of the relatively small changes in drug accumulation and cytotoxicity observed in SY5Y and 15N cells is unknown. In clinical practice, chemotherapy is typically given at maximum tolerated doses, and it is well accepted that dose reductions compromise efficacy. Cytokine therapy, which is widely used to stimulate recovery from chemotherapy-induced neutropenia, enables an increased dose intensity of only 130–220% (47). Although it is not known how an increase in resistance of 2–3-fold in *in vitro* assays may translate to patients, such an alteration in resistance may diminish the efficacy of chemotherapy. It has been shown that dose intensity correlates strongly with response, median survival, and progression-free survival in NB (48). The modest degree of protection that is detected in these *in vitro* studies is comparable to that provided by other mechanisms inherent in “cell biology,” including metabolic detoxification, damage and repair mechanisms, and cell survival mechanisms (49). Our finding that the expression of a factor such as BDNF in NB cells can partially blunt the cytotoxic effects of vinblastine suggests that the effects of neurotrophins may be a novel mechanism whereby neuroectodermal tumor cells escape damage by a chemotherapeutic agent.

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