

Downregulation of Bim by brain-derived neurotrophic factor activation of TrkB protects neuroblastoma cells from paclitaxel but not etoposide or cisplatin-induced cell death

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Chemoresistance and increased expression of TrkB and brain-derived neurotrophic factor (BDNF) are biomarkers of poor prognosis in tumors from patients with neuroblastoma (NB). Previously, we found BDNF activation of TrkB through PI3K/Akt protects NB from etoposide/cisplatin-induced cell death. In this study, the role of Bim, a proapoptotic protein, was investigated. Bim was involved in paclitaxel but not etoposide or cisplatin-induced cell death in NB cells. Pharmacological and genetic studies showed that BDNF-induced decreases in Bim were regulated by MAPK and not PI3K/Akt pathway. Both MAPK and PI3K pathways were involved in BDNF protection of NB cells from paclitaxel-induced cell death, while PI3K predominantly mediated BDNF protection of NB cells from etoposide or cisplatin-induced cell death. These data indicate that different chemotherapeutic drugs induce distinct death pathways and growth factors utilize different signal transduction pathways to modulate the effects of chemotherapy on cells.

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Neuroblastoma (NB) is a pediatric solid tumor derived from neural crest precursor cells.¹ Although NB tumors are initially sensitive to chemotherapy, they ultimately develop resistance. Understanding the signal transduction pathways that affect the sensitivity of NB cells to chemotherapy is important for developing more effective therapies. As cell lines and tumors from patients with a poor prognosis frequently express brain-derived neurotrophic factor (BDNF) and its tyrosine kinase receptor TrkB,^{2,3} we have studied the involvement of this signal transduction pathway in the sensitivity of cells to chemotherapy.

BDNF is a member of the neurotrophin family, which is important in the survival and development of the central nervous system. BDNF binds to the tyrosine kinase receptor TrkB and initiates multiple signaling cascades, including mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol 3-kinase (PI3K) pathway, and phospholipase C- γ (PLC- γ) pathways.^{4,5} BDNF promotes survival of NB cells and protects them from chemotherapy.^{6,7} Our previous studies show that TrkB activation by BDNF protects NB cells from chemotherapy via activation of the PI3K pathway.⁸ More recently, we showed that Akt, a key target of PI3K, can functionally mimic BDNF to protect NB cells from etoposide and cisplatin-induced cell death.⁹

BDNF activation of TrkB stimulates PI3K and leads to phosphorylation and activation of Akt.¹⁰ Activated Akt phosphorylates and inhibits several proapoptotic proteins, such as Bad, caspase-9 and the Forkhead transcription

factors that include FKHRL1, FKHR and AFX, leading to cell survival.^{11–15} Although several growth factors (such as IGF-I, TGF, EPO, PDGF)^{16–19} have been found to induce FKHRL1 phosphorylation via the Akt pathway, the role of Akt in BDNF regulation of FKHRL1 has not been explored. Forkhead transcription factor-induced cell death appears to be mediated by transcriptional regulation of a number of genes including Fas ligand (FasL)^{15,20} and Bim, a BH3 only domain protein that modulates the intrinsic mitochondrial death pathway.²¹ Alternative mRNA splicing generates three major isoforms of Bim: Extra-Long (BimEL), Long (BimL), and Short (BimS), with BimEL representing the predominant isoform in most tissues.²² FKHRL1-dependent increases in Bim mediate cell death upon cytokine withdrawal in a number of hematopoietic cell types²³ and Bim functions as a tumor suppressor in solid tumors of epithelial origin and lymphomas.^{24,25} Akt regulates FKHRL1-dependent transcription of Bim in T-cells²¹ and neurons,²⁶ while in other model systems regulation of Bim is independent of Akt and dependent on MAPK or mTOR activation.^{27–34}

In this study, we evaluated the regulation of Bim by BDNF/TrkB pathway and its role in the sensitivity of NB cells to chemotherapy. Using TrkB-expressing NB cells and a gene silencing approach, we found that decreases in Bim by siRNAs targeted to Bim protected NB cells from paclitaxel but not etoposide or cisplatin-induced cell death. The BDNF-induced reduction of Bim was dependent on activation of MAPK and independent from activation of PI3K/Akt/FKHRL1

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Abbreviations: BDNF, brain-derived neurotrophic factor; MAPK, mitogen-activated protein kinase; NB, neuroblastoma; PI3K, phosphatidylinositol 3-kinase

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pathway. These studies indicated that different chemotherapeutic agents induced cell death via distinct mechanisms, and growth factors used distinct signal transduction pathways to attenuate the effects of cytotoxic agents.

Results

BDNF downregulates BimEL levels. Bim is reported to be a tumor suppressor^{24,25} and a downstream target of Akt/FKHRL1.^{21,26} As our previous studies showed that PI3K/Akt pathway played an important role in BDNF/TrkB protection of NB cells from chemotherapy,^{8,9} we evaluated the role of the proapoptotic protein Bim in our model system. TB8 cells were treated with 100 ng/ml BDNF for up to 6 h (0–6 h) and BimEL, the phosphorylation of FKHRL1 (P-FKHRL1) and Akt (P-Akt, as a control of BDNF stimulation) and total FKHRL1 and Akt levels were assessed. As shown in Figure 1, BDNF activation of TrkB stimulated a reduction in BimEL levels in TB8 cells, and a rapid increase in phosphorylation of FKHRL1 and Akt. BimEL levels decreased within 5 min of BDNF stimulation, and remained depressed even after 6 h. A 5-min stimulation of BDNF induced a five and six-fold increase in P-FKHRL1 and P-Akt, respectively. The increase of FKHRL1 and Akt phosphorylation remained detectable for at least 6 h while the levels of total FKHRL1 and Akt did not change. These data indicate that the BDNF/TrkB-induced decrease in BimEL levels was temporally related to the increases in Akt and FKHRL1 phosphorylation.

Silencing of BimEL by siRNA implicates Bim in paclitaxel but not etoposide or cisplatin-induced cell death in TB8 cells. To evaluate the role of BimEL in BDNF/TrkB protection of TB8 cells from chemotherapy-induced cell death, we performed gene silencing experiments. Three different BimEL siRNA duplexes (no. 1, no. 2, no. 3) were transfected into TB8 cells and BimEL mRNA levels were examined 24 h post-transfection. The BimEL mRNA levels were decreased by more than 70% in cells transfected with BimEL siRNAs compared to cells transfected with control siRNA (Figure 2a). To evaluate if a decrease in BimEL levels by siRNA is involved in chemotherapy-induced cell death in TB8 cells, we transfected BimEL siRNA no. 2, no. 3 or a control siRNA into TB8 cells. At 48 h after transfection, the

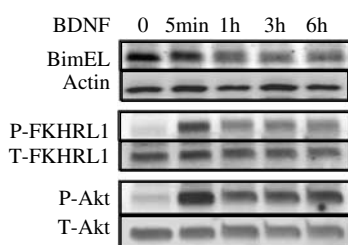


Figure 1 BDNF stimulation downregulated BimEL levels and induced phosphorylation of FKHRL1 in a time-dependent manner. TB8 cells were treated with 100 ng/ml BDNF, and then harvested at the indicated times (5 min, 1, 3, 6 h). Whole cell lysate were extracted and 30 μ g of total protein were analyzed for BimEL, P-FKHRL1 (Thr32), P-Akt (Ser 473), T-FKHRL1 and T-Akt by Western blotting. Actin was used as a loading control

cells were analyzed either for BimEL expression (Figure 2b), or for their response to different concentrations of three chemotherapeutic drugs, paclitaxel (5, 10, 20 μ g/ml), etoposide (0.25, 0.5, 1 μ g/ml) and cisplatin (0.5, 1, 3 μ g/ml) respectively (Figure 2c–e). Transfection of BimEL siRNA caused a 50% decrease in BimEL protein levels (Figure 2b) at the time cells were treated with paclitaxel, etoposide or cisplatin. In the paclitaxel-treated group, there was a 16% increase in the survival of the cells transfected with BimEL siRNA no. 2 or no. 3 which was statistically significant ($P < 0.05$) compared to the cells transfected with control siRNA (Figure 2c). However, in etoposide or cisplatin-treated groups, there was no difference in survival for TB8 cells transfected with BimEL siRNA no. 2 or no. 3 compared to cells transfected with control siRNA (Figure 2d, e). These results indicate that the decrease in BimEL levels using the BimEL siRNA altered the sensitivity of TB8 cells to paclitaxel but not etoposide or cisplatin.

BDNF protects NB cells from different types of chemotherapeutic drugs-induced cell death. To study the role of Bim in different types of chemotherapy-induced cell death in NB cells, we treated NB cells with paclitaxel, etoposide and cisplatin and evaluated changes in cell cycle distribution at multiple times after drug treatment. Figure 3a shows that from 8 to 24 h of treatment with paclitaxel (20 μ g/ml), there was an increase in the fraction of cells in the G2/M phase of the cell cycle, while by 48 h cells accumulated in the subG1 phase. These results show that after paclitaxel treatment TB8 cells arrested in the G2/M phase of the cell cycle prior to cell death. In contrast, when TB8 cells were treated with etoposide (0.5 μ g/ml, Figure 3b) or cisplatin (3 μ g/ml, Figure 3c), there was an increase in accumulation of cells in sub-G1 phase of the cell cycle within 16 h of treatment that continued at 24 and 48 h. These results indicate that although TB8 cells were sensitive to paclitaxel and etoposide or cisplatin, the response of the cells to paclitaxel was distinct from their responses to etoposide or cisplatin. Our previous results showed that BDNF protected NB cells from etoposide and cisplatin-induced cell death. Etoposide and cisplatin are both DNA damaging drugs. Paclitaxel exerts its cytotoxic effect by binding microtubules and inhibiting their depolymerization into tubulin monomers. As paclitaxel was not analyzed in our previous studies, we tested whether BDNF would protect NB cells from paclitaxel-induced cell death. TB8 cells were pretreated with BDNF (100 ng/ml, 1 h) followed by 48 h treatment with different concentrations of paclitaxel (5, 10, 20 μ g/ml). Depending on the paclitaxel dose, pretreatment with BDNF rescued up to 30% of the cells from drug induced-cell death and the effects of BDNF were statistically significant (Figure 3d, * $P < 0.05$, ** $P < 0.01$).

Silencing of BimEL mimics the ability of BDNF to protect TB8 cells from paclitaxel-induced cell death. As the cell cycle profiles of TB8 cells were distinct after treatment with paclitaxel and etoposide, we evaluated whether BDNF or silencing of BimEL would change the response of cells to paclitaxel and etoposide. We restricted these studies to etoposide since the response of the TB8 cells to etoposide

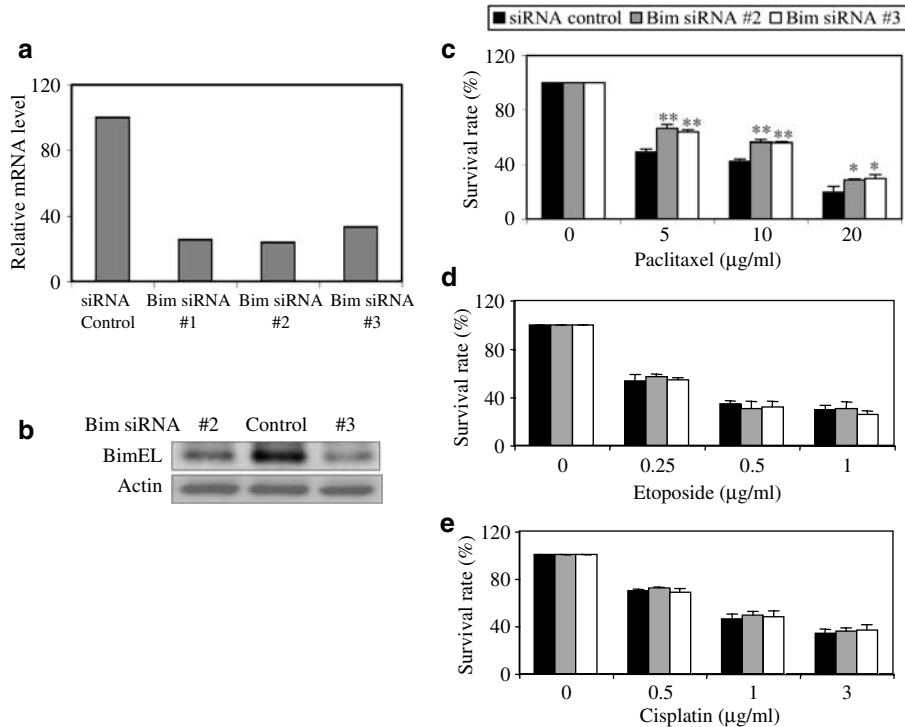


Figure 2 Silencing of BimEL by siRNA was involved in paclitaxel-induced cell death, but not etoposide or cisplatin-induced cell death in TB8 cells. 2 μg BimEL siRNA no. 1, no. 2, no. 3 or siRNA control were transfected into TB8 cells by nucleofector devices, respectively. At 24 h after transfection total RNA was prepared for RT-PCR. BimEL mRNA levels were detected and normalized by actin control (a). 2 μg BimEL siRNA no. 2, no. 3 or siRNA control were transfected into TB8 cells by nucleofector devices, respectively, 48 h after transfection, whole cell lysate were extracted and analyzed by Western blotting for BimEL and actin (b), and some TB8 cells were treated with paclitaxel (5, 10, 20 $\mu\text{g}/\text{ml}$, 48 h) (c), etoposide (0.25, 0.5, 1 $\mu\text{g}/\text{ml}$, 24 h) (d) or cisplatin (0.5, 1, 3 $\mu\text{g}/\text{ml}$, 24 h) (e). MTS assay was used to detect cell survival. Survival rate in every condition was normalized to the no-chemotherapeutic drug control within every group. Bars, S.D. Significance was tested by ANOVA, Bonferroni was used as post-test. * $P < 0.05$, ** $P < 0.01$ versus siRNA control cells

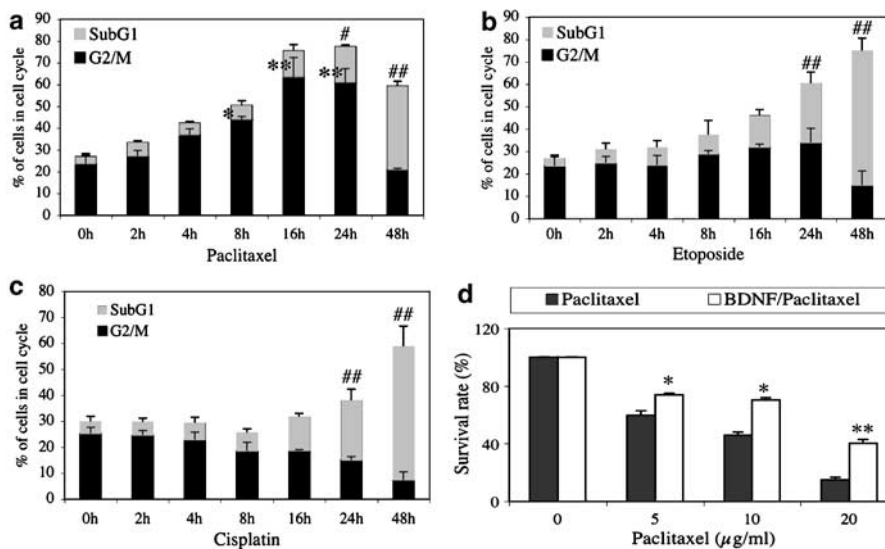


Figure 3 TB8 cells responded differently to paclitaxel and etoposide or cisplatin treatment. TB8 cells were first treated with paclitaxel (20 $\mu\text{g}/\text{ml}$) (a), etoposide (0.5 $\mu\text{g}/\text{ml}$) (b) or cisplatin (3 $\mu\text{g}/\text{ml}$) (c) for the indicated time (2, 4, 8, 16, 24, 48 h), then stained with 50 $\mu\text{g}/\text{ml}$ propidium iodide and analyzed by flow cytometry. The percentages of cells in sub-G1 and G2/M phases of the cell cycle are analysed. Bars, S.D. Significance was tested by ANOVA followed by a Dunnett post-test to compare the drug-treated group at each time point versus the control group. # $P < 0.05$, ## $P < 0.01$ for SubG1 analysis, * $P < 0.05$, ** $P < 0.01$ for G2/M analysis. (d) TB8 cells were pretreated with BDNF (100 ng/ml) for 1 h, and then treated with paclitaxel (5, 10, 20 $\mu\text{g}/\text{ml}$) for 48 h. MTS assay was used to detect cell survival. Survival rate in every condition was normalized to the no-paclitaxel control within every group. Bars, S.D. * $P < 0.05$, ** $P < 0.01$ versus paclitaxel-treated cells

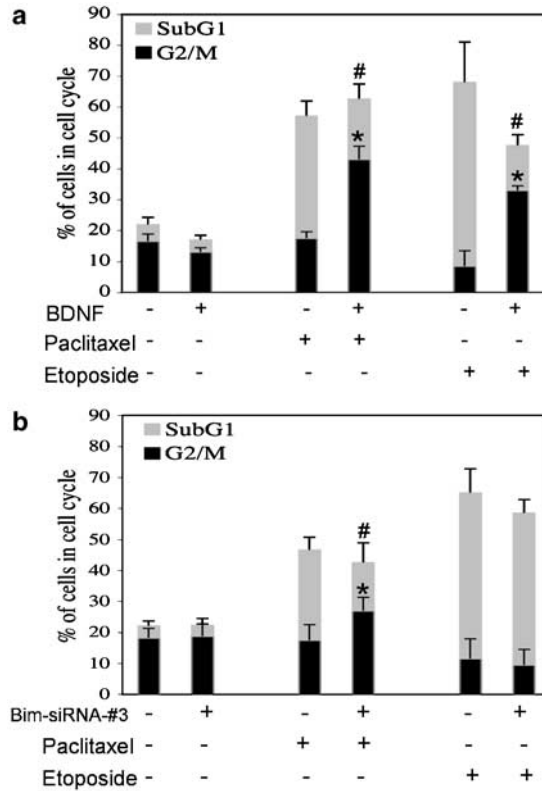


Figure 4 Silencing of BimEL by siRNA mimicked BDNF protection of TB8 cells from paclitaxel-induced cell death. TB8 cells were pretreated with BDNF (100 ng/ml) for 1 h (a), or transfected with 2 μ g Bim siRNA #3 or siRNA control, and cultured for 48 h (b), then treated with paclitaxel (20 μ g/ml) or etoposide (0.5 μ g/ml) for 48 h. The cells were stained with 50 μ g/ml propidium iodide and analyzed by flow cytometry. The percentages of cells in G2/M and sub-G1 phases of the cell cycle are analyzed. Bars, S.D. # $P < 0.05$ for SubG1, * $P < 0.05$ for G2/M, versus paclitaxel- or etoposide-treated cells

and cisplatin were indistinguishable. We treated the TB8 cells with BDNF (1 h, 100 ng/ml) (Figure 4a) or transfected with Bim siRNA no. 3 (Figure 4b), followed by treatment with paclitaxel (20 μ g/ml) or etoposide (0.5 μ g/ml) for 48 h. Pretreatment with BDNF decreased the paclitaxel and etoposide-induced accumulation of cells in the sub-G1 fraction and increased the G2/M fraction of the cell cycle at 48 h (Figure 4a). Silencing of BimEL by Bim siRNA decreased the paclitaxel-induced accumulation of cells in the sub-G1 fraction and increased the G2/M fraction of the cell cycle at 48 h. However silencing of BimEL levels had no effect on the cell cycle changes induced by etoposide treatment (Figure 4b). These results indicate that silencing of BimEL by Bim siRNA mimicked the ability of BDNF to protect TB8 cells from paclitaxel but not etoposide-induced cell death.

BDNF/TrkB-induced decreases in BimEL is mediated by MAPK but not PI3K pathway. Our previous finding that the PI3K/Akt pathway modulates cell sensitivity to etoposide/cisplatin-induced cell death^{8,9} coupled with a lack of involvement of Bim in this process raises questions as to which signaling pathway mediated the BDNF/TrkB-induced

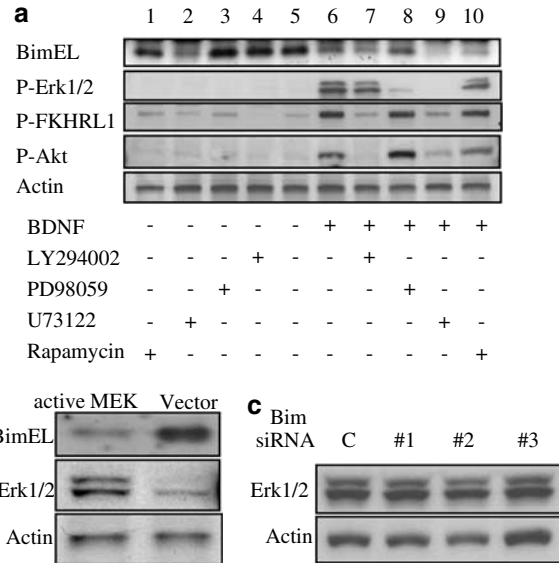


Figure 5 BDNF downregulated BimEL level via the MAPK pathway. (a) TB8 cells were cultured in RPMI 1640 containing 10% FBS for 24 h, and then treated with different kinase inhibitors, PI3K pathway inhibitor-LY294002 (20 μ M), MAPK pathway inhibitor-PD98059 (40 μ M), PLC- γ pathway inhibitor-U73122 (20 nM), and mTOR pathway inhibitor-rapamycin (100 μ M) for 1 h followed by 100 ng/ml BDNF stimulation for 1 h. 30 μ g of protein lysate was analyzed by Western blotting for BimEL, P-Erk1/2 (Thr 202/Tyr204), P-FKHRL1 (Thr32), P-Akt (Ser473). Actin was used as loading control. (b) 8 μ g GFP and either 12 μ g active MEK or 12 μ g empty vector were cotransfected into TB8 cells by electroporation. After 48 h transfection whole cell lysate were extracted and analyzed by Western blotting for BimEL, P-Erk1/2 and actin. (c) 2 μ g BimEL siRNA no. 1, no. 2, no. 3 or siRNA control were transfected into TB8 cells by nucleofector devices, respectively. At 48 h after transfection whole cell lysate were extracted and analyzed by Western blotting for Erk1/2 and actin

decreases in BimEL. TB8 cells were pretreated with different signal transduction pathway inhibitors (the PI3K inhibitor LY294002, the MAPK inhibitor PD98059, the PLC- γ inhibitor U73122 and the mTOR inhibitor rapamycin), and then stimulated with BDNF. Except U73122, all the other inhibitors did not affect Bim levels (Figure 5a, lane 1, 3 and 4 versus control lane 5). BDNF (100 ng/ml) caused a 40% decrease in BimEL levels (Figure 5a, control-lane 5 versus BDNF-lane 6). Pretreatment of TB8 cells with the MAPK inhibitor PD98059 slightly reversed the BDNF-induced reduction in BimEL levels (Figure 5a, lane 8 versus lane 6). In contrast, the PI3K inhibitor LY294002 (Figure 5a, lane 7 versus lane 6), PLC- γ inhibitor U73122 (Figure 5a, lane 9 versus lane 6), and the mTOR inhibitor rapamycin (Figure 5a, lane 10 versus lane 6) failed to significantly alter the BDNF-induced decrease in BimEL levels. In the same samples, we evaluated which signaling pathways mediated BDNF-induced phosphorylation of Akt and FKHRL1. Pretreatment of TB8 cells with LY294002 or U73122 blocked BDNF-induced phosphorylation of FKHRL1 and Akt. For Erk1/2, a target in the MAPK pathway (which we used to test the specificity of MAPK inhibitor), BDNF stimulation induced the phosphorylation of Erk1/2, which was blocked by pretreatment with PD98059 and U73122. Pretreatment with rapamycin did not affect BDNF-induced phosphorylation of FKHRL1, Akt or Erk1/2.

We further examined the role of the MAPK pathway in BDNF/TrkB regulation of BimEL by transfecting a constitutively active MEK or a control empty vector into TB8 cells. Activation of MEK induced phosphorylation of its downstream target Erk1/2 in the cells transfected with the constitutively active MEK expression vector compared to empty vector-transfected cells (Figure 5b). At the same time, the BimEL levels decreased in constitutively active MEK-transfected cells compared to empty vector-transfected cells (Figure 5b). This indicated that activation of MAPK pathway was sufficient to reduce BimEL levels, and this result mimicked the regulation of BimEL by BDNF activation of TrkB. Meanwhile, when we silenced BimEL by BimEL siRNA, Erk1/2 levels did not change (Figure 5c). This indicates that BimEL was downstream of Erk1/2 in the MAPK pathway. Using a pharmacologic and a genetic approach, these data indicate that the MAPK pathway mediated the BDNF/TrkB-induced decrease in BimEL levels.

BDNF protects NB cells from paclitaxel-induced cell death via both PI3K and MAPK pathways. To study the involvement of PI3K and MAPK pathways in the BDNF

protection of NB cells from paclitaxel-induced cell death, we pretreated the cells with MAPK inhibitor PD98059 or PI3K inhibitor LY294002 1 h before BDNF stimulation, and then treated with paclitaxel (20 $\mu\text{g/ml}$) for 48 h. Pretreatment with BDNF increased the cell survival from 26 to 58% after paclitaxel treatment, so BDNF protected 32% of the cells from paclitaxel-induced cell death. Administration of PD98059 or LY294002 before BDNF stimulation blocked 13 or 20% of BDNF protection of the cells from paclitaxel-induced cell death, respectively (Figure 6a). To complement the pharmacologic approach, we used a genetic approach and found that the survival of the cells transfected with constitutively active MEK was 14% higher than that of the cells transfected with empty vector (Figure 6b). These results indicate that both the PI3K and MAPK pathways were involved in the BDNF protection of TB8 cells from paclitaxel-induced cell death. To investigate in more detail if MAPK pathway was involved in BDNF protection on TB8 cells from etoposide and cisplatin-induced cell death, we either pretreated the cells with MAPK inhibitor PD98059 (40 μM , 1 h) before BDNF stimulation, or transfected the cells with constitutively active MEK or empty vector before etoposide

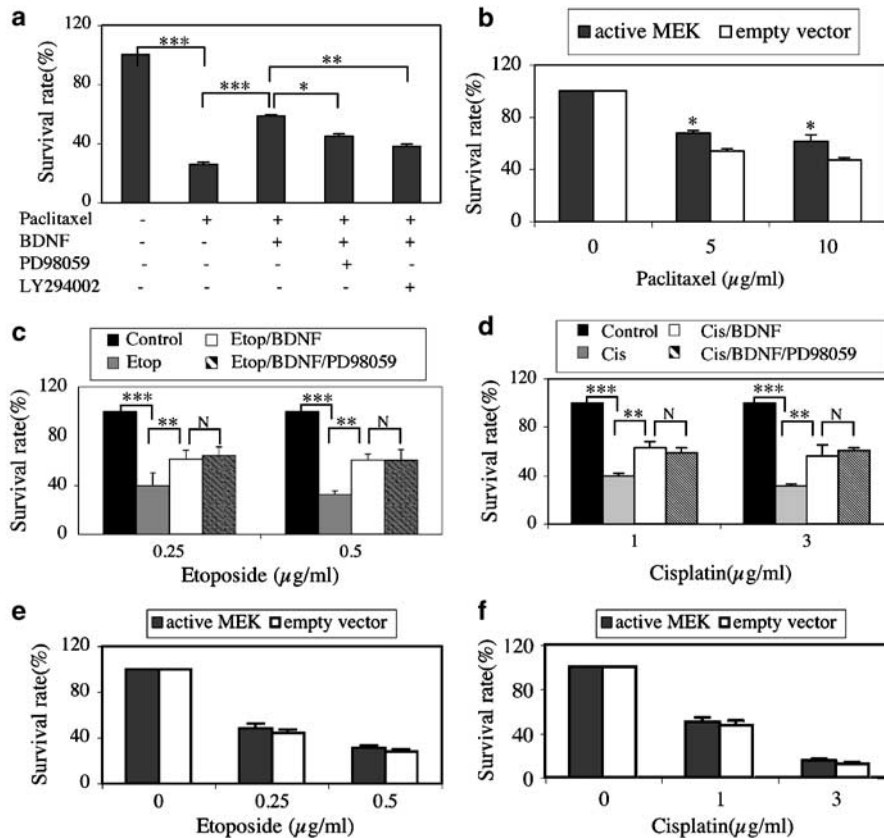


Figure 6 Both MAPK and PI3K pathways were involved in BDNF protection of NB cells from paclitaxel-induced cell death. After the indicated treatment of TB8 cells, MTS assay was used to detect cell survival. Survival rate in every condition was normalized to the no-chemotherapeutic drug control within every group. Bars, S.D. (a) TB8 cells were pretreated with MAPK inhibitor PD98059 (40 μM , 1 h) or PI3K inhibitor LY294002 (20 μM , 1 h) before BDNF stimulation (100 ng/ml, 1 h), and then treated with paclitaxel (20 $\mu\text{g/ml}$, 48 h). Significance was tested by ANOVA, Bonferroni was used as post test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (b, e and f) TB8 cells were co-transfected with 8 μg GFP and either 12 μg active MEK or 12 μg empty vector and cultured for 48 h, then treated with paclitaxel (5, 10 $\mu\text{g/ml}$, 48 h) (b), etoposide (0.25, 0.5 $\mu\text{g/ml}$, 24 h) (e) or cisplatin (1, 3 $\mu\text{g/ml}$, 24 h) (f) respectively. * $P < 0.05$, versus empty vector transfected cells. (c and d) TB8 cells were pretreated with PD98059 (40 μM , 1 h) followed by BDNF stimulation (100 ng/ml, 1 h), and then treated with etoposide (0.25, 0.5 $\mu\text{g/ml}$) (c) or cisplatin (1, 3 $\mu\text{g/ml}$) (d) for 24 h, respectively. Significance was tested by ANOVA, Bonferroni was used as post-test. ** $P < 0.01$, *** $P < 0.001$, N: no statistical difference

(0.25, 0.5 $\mu\text{g/ml}$) or cisplatin (1, 3 $\mu\text{g/ml}$) treatment. Pretreatment of the cells with PD98059 did not affect the ability of BDNF to protect TB8 cells from etoposide or cisplatin-induced cell death (Figure 6c, d). Moreover, transfection of constitutively active MEK into TB8 cells did not alter the survival of the cells after etoposide or cisplatin treatment (Figure 6e, f). These data indicate that the MAPK pathway was not involved in BDNF protection of NB cells from etoposide or cisplatin-induced cell death.

To extend our studies, we established another TrkB-expressing cell line by transfecting pBPSTR1-TrkB⁸ to the N-myc amplified NGP NB cell line and isolating a puromycin resistant clone (NGP-TrkB). Like the TB8 cells,⁸ when cells were cultured in the absence of tetracycline (TET) there was an increase in TrkB levels compared to cells cultured in the presence of TET (Figure 7a). BDNF treatment of NGP-TrkB cells caused a 50% decrease in BimEL levels. Pretreatment with the MAPK inhibitor PD98059, but not the PI3K inhibitor LY294002, prior to BDNF stimulation, blocked the decrease of BimEL (Figure 7b). BDNF-induced phosphorylation of Akt

was blocked by LY294002, but not by PD98059 (Figure 7b). NGP-TrkB cells were sensitive to paclitaxel and etoposide treatment and pretreatment with BDNF resulted in a significant increase in the survival of cells from paclitaxel or etoposide treatment ($*P < 0.05$, $**P < 0.01$) (Figure 7c, d). Consistent with our studies in TB8 cells, BDNF treatment protected 23 or 29% of NGP-TrkB cells from paclitaxel or etoposide-induced cell death respectively. Pretreatment with PD98059 before BDNF stimulation blocked 11% of BDNF protection of cells in paclitaxel-treated cells (Figure 7e), but had no effect in etoposide-treated cells (Figure 7f). Pretreatment with LY294002 before BDNF stimulation blocked 16 or 19% of BDNF protection of cells from paclitaxel or etoposide-induced cell death (Figure 7e, f) respectively.

Discussion

Our results showed that silencing of BimEL by siRNA protected TB8 cells from paclitaxel-induced cell death, but not etoposide or cisplatin-induced cell death. This indicates for

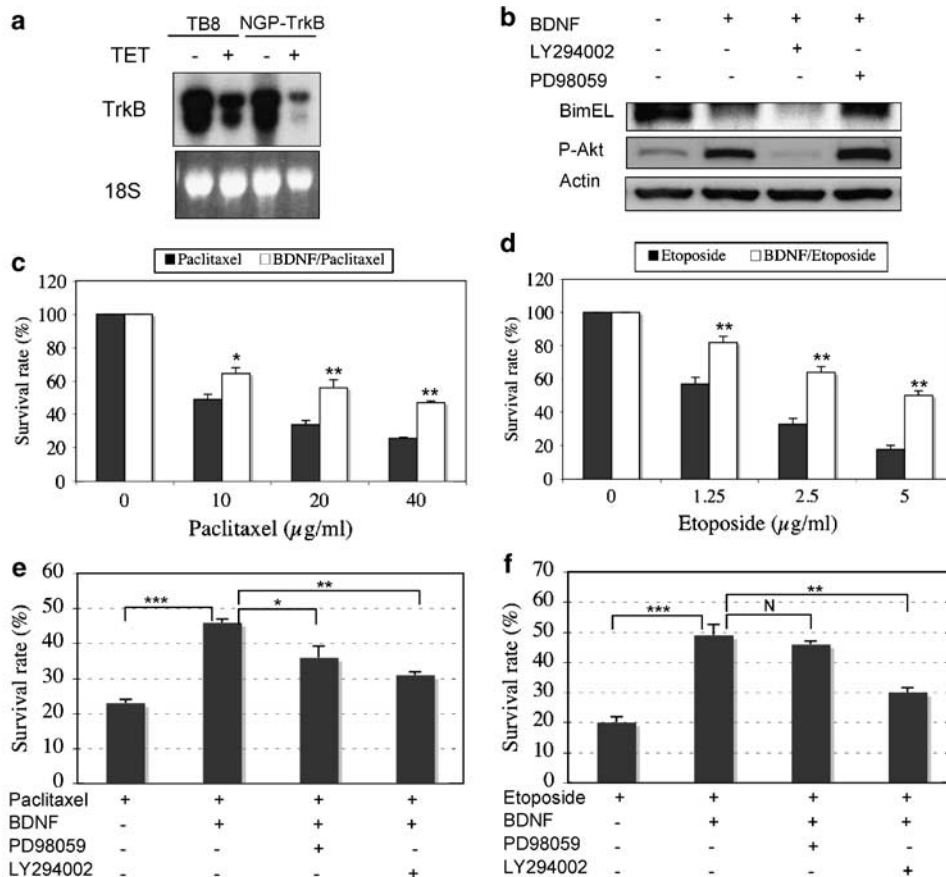


Figure 7 MAPK pathway was involved in BDNF protection from paclitaxel-induced cell death in NGP-TrkB cells. (a) TrkB mRNA expression in TB8 and NGP-TrkB cells was assessed by Northern Blot analysis as described in 'Materials and methods'. 18 s RNA served as a loading control. (b) NGP-TrkB cells were treated with PD98059 (40 μM) or LY294002 (20 μM) for 1 h followed by 100 ng/ml BDNF stimulation for 30 min. Thirty μg of protein lysate were analyzed by Western blotting for BimEL, P-Akt(Ser473) and actin. (c and d) NGP-TrkB cells were pretreated with BDNF (100 ng/ml) for 1 h, and then treated with paclitaxel (10, 20, 40 $\mu\text{g/ml}$) or etoposide (1.25, 2.5, 5 $\mu\text{g/ml}$) for 48 h. MTS assay was used to detect cell survival. Survival rate in every condition was normalized to the no-chemodrug control within every group. Bars, S.D. $*P < 0.05$, $**P < 0.01$ versus paclitaxel or etoposide-treated cells. (e, f) NGP-TrkB cells were pretreated with PD98059 (40 μM , 1 h) or LY294002 (20 μM , 1 h) before BDNF stimulation (100 ng/ml, 1 h), and then treated with paclitaxel (40 $\mu\text{g/ml}$) or etoposide (5 $\mu\text{g/ml}$) for 48 h. MTS assay was used to detect the cell survival. Survival rate in every condition was normalized to the no-chemodrug control within every group. Bars, S.D. Significance was tested by ANOVA, Bonferroni was used as post-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, N: no statistical difference

the first time that the role of BimEL in the BDNF/TrkB protection of NB cells is drug-dependent. Etoposide and cisplatin are DNA damaging drugs; etoposide inhibits DNA topoisomerase II, thereby inhibiting DNA synthesis, while cisplatin binds to DNA and interferes with its repair mechanism. Both interventions eventually lead to cell death. Conversely, paclitaxel stabilizes microtubules, activating the spindle assembly checkpoint and mitotic arrest.³⁵ Our cell cycle analysis showed that TB8 cells arrested in the G2/M phase prior to cell death when treated with paclitaxel. This is very similar to the cell cycle changes noted in breast cancer cells after paclitaxel treatment.³⁶ Considering the known proapoptotic mechanism of Bim, this may be why Bim plays a role in paclitaxel-induced cell death. Bim is thought to induce cell death by binding to LC8 cytoplasmic dynein light chain where it is sequestered with the microtubule-associated dynein motor complex in healthy cells.³⁷ Certain apoptotic stimuli disrupt the interaction between LC8 and the dynein motor complex freeing Bim to translocate together with LC8 to Bcl-2 and neutralize its anti-apoptotic activity.³⁷ In our present study, when the Bim levels were reduced by Bim siRNA, the accumulation of cells in sub-G1 phase induced by paclitaxel treatment decreased. Similarly, transfection of Bim siRNA into MCF-7 breast cancer cells inhibited apoptosis induced by paclitaxel treatment.³⁶ However, silencing of BimEL had no effect on the accumulation of TB8 cells in the sub-G1 portion of the cell cycle after treatment with etoposide or cisplatin. This indicates that Bim levels do not play a role in modulating etoposide or cisplatin-induced cell death in NB cells and this finding may also be relevant to other tumor types.

Initially, we had hypothesized that Bim would be an important mediator of the survival of NB cells after BDNF activation of TrkB via the PI3K/Akt pathway. However, our pharmacologic and genetic experiments indicated that the BDNF-induced reduction in Bim levels was mediated by activation of MAPK, not the PI3K/Akt pathway. As a BH3-only proapoptotic protein, Bim is expressed in hematopoietic, epithelial, neuronal and germ cells.³⁸ It is predominantly regulated by FKHRL1,^{21,23–26} and the molecular mechanism associated with FKHRL1 regulation has largely been characterized in response to growth factor signaling via the evolutionarily conserved PI3K/Akt survival signaling pathway.^{16–19,39} Using pharmacological inhibitors we found that FKHRL1 was regulated by the PI3K pathway. This is consistent with what has been reported for BDNF activation of endogenous TrkB receptors in SY5Y NB cells by Zhu *et al.*⁴⁰ Moreover, a PI3K inhibitor did not block the BDNF-induced reduction of Bim. This indicates that in our cell model system, the downregulation of Bim by BDNF is independent of PI3K, Akt and FKHRL1. Although Bim is reported to be regulated mainly via the PI3K/Akt/FKHRL1 pathway in a number of cell lines,^{21,23–26} there is increasing evidence that Bim regulation is complex and regulated by multiple growth factor signaling pathways which are cell-type specific.^{26–34} In our study, transfection of constitutively active MEK into NB cells decreased Bim levels and a pharmacological inhibitor of the MAPK pathway blocked the BDNF-induced reduction in Bim levels. This indicates that Bim is regulated by the MAPK pathway upon BDNF activation of TrkB in NB cells. The MAPK pathway is also important in the NGF inactivation of BimEL in

PC12 cells.⁴¹ In that study, BimEL is inactivated via an early phosphorylation event while an NGF induced decrease in BimEL levels occurs over several days. In our studies, BDNF/TrkB stimulates a decrease in BimEL levels that occurs within 1 h of treatment. The difference in regulation of BimEL may be due to differences in the cell type, the activation of different Trk signaling paths by their ligands. Recent evidence indicates that activation of TrkA leads to distinct changes compared to activation of TrkB in neuroblastoma cells.⁴²

In identifying the signaling pathways that mediate the BDNF/TrkB protection of NB cells from paclitaxel, etoposide or cisplatin, we found that both the PI3K and MAPK pathways protected NB cells from paclitaxel-induced cell death, while only the PI3K pathway protected cells from etoposide and cisplatin-induced cell death. The involvement of MAPK pathway in paclitaxel-induced cell death is consistent with our findings that Bim was regulated by BDNF via the MAPK pathway and Bim only altered the response of NB cells to paclitaxel-induced cell death signals. Our finding is consistent with the report by Tan *et al.*²⁴ in which MAPK pathway confers resistance to paclitaxel that is dependent on functional inactivation of Bim. This indicates that the efficacy of paclitaxel therapy may be dependent on the activation status of the MAPK pathway and Bim levels in certain tumors.^{24,35,36,43} The involvement of PI3K pathway in both etoposide/cisplatin- and paclitaxel-induced cell death indicated that the PI3K pathway still plays an important role in the BDNF protection of NB cells from chemotherapy. So our current studies are aimed at identifying the downstream targets of the PI3K pathways that mediate the BDNF protection of NB from chemotherapy. Such studies are important as more and more inhibitors of signal transduction pathways are being combined with different chemotherapeutic regimens.⁴⁴ The more restricted or specific the targets are, the more effective and less cytotoxic the treatment may be. In the future we will also assess the effects of different inhibitors in our *in vivo* NB models.

To summarize, our data demonstrate that although BDNF/TrkB can protect NB cells from cell death induced by different chemotherapeutic drugs, the mechanism by which BDNF activation of TrkB protects cells depends on the mechanism of cell death induced by the chemotherapeutic drugs. This study indicates that to more effectively utilize small molecule inhibitors of survival signaling pathways with traditional chemotherapy will require a detailed blueprint of the chemotherapy-associated apoptotic pathways and survival pathways utilized by different cell types. In the clinic these data will lead to better understanding of how to combine chemotherapeutic drugs with different signal transduction pathway inhibitors, to improve the efficacy of therapeutic regimens.

Materials and Methods

Cells and cell culture. Stable TrkB expressing cell line-TB8 cells⁴⁵ and NGP-TrkB cells were cultured in RPMI 1640 (Mediatech Inc., Herndon, VA, USA) containing 10% fetal bovine serum (FBS), 2 mM glutamine, and antibiotics at 37°C in 5% CO₂ incubator as described previously.⁸ To maintain selection pressure the TB8 and NGP-TrkB cells were cultured in puromycin (0.5 µg/ml) (Sigma-Akdrich Inc., Steinheim, Germany).

DNA Vector Constructions. Constitutively active MEK and empty vector were provided by Dr. Natalie G Ahn.⁴⁶

Reagents. Recombinant human BDNF was obtained from PeproTech Inc. (Rocky Hill, NJ, USA). LY294002, PD98059, U73122 and Rapamycin were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-FKHRL1 (Thr32), anti-Akt, anti-phospho-Akt (Ser473), anti-phospho-Erk1/2 (Thr202/Tyr204) and anti-Erk1/2 were obtained from Cell Signaling Technology, while anti-FKHRL1 (Upstate Biotechnology, Lake Placid, NY, USA), anti-Bim (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and actin (Abcam Ltd Cambridge, UK) were obtained from indicated sources. Paclitaxel, etoposide and cisplatin were purchased from Sigma (St Louis, MO, USA).

Protein analysis. Proteins were detected by Western blotting with indicated antibodies as described previously.⁸ Briefly, protein lysates (30 μ g) were analyzed by SDS-PAGE and transferred to nitrocellulose. The indicated primary antibodies were diluted according to manufactures's recommendations and detected with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (1:2000 dilution).

Transfections. TB8 (8×10^6) cells in 400 μ l media were co-transfected with green fluorescent protein (GFP, 8 μ g) and either constitutively active MEK (12 μ g), or empty vector control (12 μ g) by electroporation at 250 mF. Bim siRNA and siRNA control (Dharmacon Inc., Lafayette, CO, USA) were transfected into TB8 (2×10^6) cells by Nucleofector device from Amaxa Biosystems (Allemanne, Germany), using cell line nucleofector™ kit V and program A-023.

Real-time quantitative PCR. Total RNA was isolated using a Qiagen Extraction procedure.⁸ Equal amounts of total RNA (2 μ g) were reverse-transcribed using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen), and the resulting first strand cDNA was diluted and used as template in the real-time quantitative-PCR analysis. All measurements were performed in triplicate. Actin served as internal control and was used to normalize the variances in input cDNA. The following gene-specific primer pair was designed using the ABI Primer Express software: Bim-sense 5'-GCCCAAGAGTTGCGGCGTA-3'; Bim-antisense 5'-CACACCAGGCGGACAATGTAA-3'. The specificity of each primer was determined using NCBI BLAST module. Detection of Bim expression was performed with SYBR Green (Applied Biosystems) and an ABI PRISM 7700 Sequence Detection System (Applied Biosystems), using the relative standard curve method.

Gene silencing with small interfering RNAs. We designed and purchased three small interfering RNA (siRNA) oligonucleotides from Dharmacon Research Inc. (Lafayette, CO, USA). TB8 cells were cultured 48 h before transfection. Oligonucleotides (2 μ g) were transfected into TB8 cells using an Amaxa nucleofector device, program A-023 and cell line Nucleofector™ kit V from Amaxa Biosystems. At 24 h after transfection, cells were harvested for extraction of RNA. At 48 h after transfection cells were collected for Western Blot, or treated with chemotherapeutic drugs. The oligonucleotides were as follows: no. 1 Bim sense 5'-UUACGACUGUACGUACA(dTdT)-3'; no. 1 Bim antisense 5'-UGUACGUAACAGUCGUAA(dTdT)-3'. no. 2 Bim sense 5'-GCAACCUUCUGAUGUAAGU(dTdT)-3'; no. 2 Bim antisense 5'-ACUACAUVAGAAAGGUUGC(dTdT)-3'. no. 3 Bim sense 5'-GACAGACCACAAGGUAAU(dTdT)-3'; no. 3 Bim antisense 5'-AUUACCUUGGUCUCUGUC(dTdT)-3'.

Treatments. To study the inhibitory effect of LY294002, PD98059, U73122, and Rapamycin on cell signaling, NB cells were plated into six-well plates in RPMI 1640 containing 10% FBS and incubated for 24 h. Cells were pretreated with different inhibitors for 1 h, then stimulated with 100 ng/ml BDNF or media control for 1 h. To study the cell survival after treatment with chemotherapeutic drugs, NB cells were either pretreated with LY294002 or PD98059 followed by BDNF, or pretreated with only BDNF, or cultured for 48 h after transfection, and then treated with indicated chemotherapeutic drugs (paclitaxel, etoposide and cisplatin) for 24 or 48 h. MTS assay was used to detect the cell survival.

Cell survival analysis. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay (MTS assay) was performed according to the manufacturer's specification. The percentage of cell survival (survival rate) was calculated by dividing the absorbance value of the treated samples by the absorbance value of the untreated control within every group. All experiments were repeated two to three times.

Cell cycle analysis. Cell cycle analysis was performed using propidium iodide staining. TB8 cells were harvested, washed with PBS twice and stained with 50 μ g/ml propidium iodide containing 5 μ g/ml DNase-free RNase A for 1 h at room temperature, and analyzed by flow cytometry with a FACScan and Cell Quest software (BD Biosciences).

Northern blot analysis. RNA isolation and hybridization were performed as described previously.⁸ Total RNA (12 μ g) harvested from TB8 and NGP-TrkB cells cultured in the absence and presence of tetracycline (1 μ g/ml) for 3 days were electrophoresed in 1% agarose-6% formaldehyde gels. Gels were stained with 2 μ g/ml ethidium bromide and 18 S RNA served as a loading control. Hybridization was performed with ³²P-labeled insert DNA isolated from a plasmid containing rat-TrkB.

Statistics. Significance was tested by ANOVA using GraphPad InStat software for Macintosh. Bonferroni or Dunnett were used as post-test. Two group comparisons were performed with Student's t-test. A P-value of <0.05 was considered as statistically significant. Data were expressed as means \pm S.D.

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