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ORIGINAL ARTICLE

Differential roles of Trk and p75 neurotrophin receptors in tumorigenesis and chemoresistance ex vivo and in vivo

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Abstract The neurotrophin receptors TrkA (NGF receptor) and TrkC (NT-3 receptor) have been shown to be important in staging disease and predicting progression and drug response for various neoplasias such as neuroblastoma, medulloblastoma and prostate cancer. Less is known about the role of the p75 neurotrophin receptor in cancer, but it influences metastatic potential in glioblastoma. To determine the effect of each neurotrophin receptor or co-receptor expression in tumorigenesis, we examined PC12 pheochromocytomas. PC12 wild type ($TrkA^{+}$, $p75^{++}$) were compared to three PC12-derived cell lines expressing varying levels of TrkA or TrkC and/or p75. Growth rates, tumorigenic potential ex vivo and in vivo, and chemotherapeutic drug response profiles differed depending on the neurotrophin receptor phenotype. The ability of neurotrophins to rescue cells from doxorubicin or cisplatin induced cell death also varied depending on phenotype. Thus, unique neurotrophin receptor tumor profiles may determine tumor aggressiveness and chemoresistance. This work may help to develop tailored therapies for specific tumor phenotypes by combining traditional chemotherapy with neurotrophin receptor modulators.

Keywords Neurotrophin · Receptor · Trk · p75 · Neural crest tumors · Growth kinetics · Tumorigenic potential · Drug resistance

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Introduction

Neurotrophins consist of a family of growth factor proteins involved in the regulation of neuronal survival, maintenance, death or differentiation. The best-studied members of the neurotrophin family include nerve growth factor (NGF), brain-derived growth factor (BDNF), and neurotrophin-3 (NT-3). Neurotrophins mediate their actions by interacting with two different classes of cell surface receptors: selective Trk tyrosine kinase receptors and the "shared" p75 receptor. Each neurotrophin binds with high affinity to Trk receptors: NGF activates TrkA, and BDNF interacts with TrkB. Although NT-3 primarily acts on TrkC, it also binds to TrkA [6, 31]. All neurotrophins bind to the common low affinity p75 receptor, member of the tumor necrosis factor superfamily (TNFR) of receptors [11].

As reported for many receptor tyrosine kinases (RTKs), over-expression of TrkA or TrkC leads to ligand-independent activation and transformation [23, 24, 29] and enhanced cell survival and/or growth. On the other hand, the ligand-dependent (and ligand-independent) activities of these receptors are also associated with cellular differentiation and apoptosis [11, 24, 32]. Mechanisms of p75 signal

transduction in the presence of Trk receptors remain unclear to date, but it is known to signal independently to regulate either apoptosis or survival in a p53-dependent manner [1].

Disturbances in neurotrophin signaling or receptor activity have been associated not only to various neurodegenerative disorders but also to several cancers of neuronal (e.g. neuroblastomas, medulloblastomas, gliomas) and of non-neuronal origin (e.g. breast, prostate). Neuroblastomas, pediatric tumors originating from precursor neural crest cells [2], are thought to undergo neoplastic transformation due to a dysregulation of growth factor signaling or to lack of normal apoptosis. Retrospective studies have linked high TrkA expression (and low TrkC levels) to favorable neuroblastoma prognosis. In contrast, unfavorable tumor outcomes were characterized by downregulation of TrkA and upregulation of TrkB expression [24]. Similar neurotrophic mechanisms are thought to control the development and outcome of medulloblastomas, where favorable tumors express high levels of TrkC, and the loss of TrkC being a poor prognostic factor [29], and ectopic expression of TrkA sensitizing these tumors to apoptosis [4].

The expression level of Trk receptors is therefore a useful prognostic indicator for clinical progression of these tumors [23] and possibly to understand the resistance of these tumors to some forms of chemotherapy. Recently, p75 expression was reported to be a marker of metastatic potential in glioblastoma [15], with autophagic function upon expression of TrkA [10]. The underlying role of p75 in disease remains to be elucidated due to its varying intrinsic roles and its regulatory functions upon Trks. Moreover, the actions of p75 depend on ligand, cell type, developmental stage and Trk receptor co-expression [13], and this further contributes to the complexity of its character.

We aimed to clarify some of the actions of TrkA, TrkC, and p75 in tumorigenesis. We characterized the in vitro and in vivo growth kinetics and tumorigenic potentials of rat pheochromocytoma PC12-derived tumors with distinct neurotrophin expression profiles, where each receptor is expressed alone or in co-expression. Each cell type had unique growth kinetics, with cells lacking p75 or TrkA doubling faster and having more aggressive tumorigenic potentials in vitro, whereas cells co-expressing TrkA or TrkC with p75 have less aggressive phenotypes. In vivo tumorigenic profiles were also determined. Each cell type also had unique sensitivity profiles to doxorubicin and cisplatin, two commonly used chemotherapeutics. Moreover, administration of neurotrophins rescued specific cell types from doxorubicin-induced death but only PC12-wt were rescued from cisplatin-induced death. Our studies may therefore help develop a therapeutic rationale tailored to specific tumors expressing specific neurotrophin receptor phenotypes.

Materials and methods

Chemicals and reagents

RPMI 1640, geneticin (G418) penicillin/streptomycin solution (P/S), HEPES buffer and L-glutamine were all purchased from Gibco. Bovine serum albumin crystalline fraction V and fetal bovine serum (FBS) were purchased from Sigma while protein-free hybridoma medium came from PFHM-II, Life Technologies Inc. NGF (Prince Labs) and human recombinant NT-3 (ProSpec-Tany) were prepared as 4 μM stock solutions in 1× PBS with 0.5% BSA. Doxorubicin hydrochloride (Sigma) solution was prepared as 2 mg/ml in 0.9% sodium chloride and *cis*-Diammineplatinum(II) dichloride (Sigma) was prepared as 3 μM stock in HBSS (Gibco).

Cell lines and culture

Rat pheochromocytoma PC12-wt cells and several variants were used (relative receptor expression levels are indicated by $^{+}$ and $^{-}$). PC12-wt (TrkA^{+}, p75^{++}) express low levels of rat TrkA (~2,000 receptors per cell) and moderate levels of p75 receptors (~75,000 receptors per cell). PC12-p75^{-} (TrkA^{+}, p75^{-}) are a variant of PC12-wt having lost detectable p75 receptor mRNA and protein expression but retaining TrkA expression and NGF responsiveness. NNr5-wt cells (TrkA^{-}, p75^{++}) are PC12-wt variants with no detectable TrkA expression, but that have conserved the same density of p75 receptor as the parental PC12-wt cell line. NNr5-TrkC (TrkC^{+}, p75^{++}) were stably transfected with human TrkC cDNA under geneticin selection (0.5 mg/ml). All cells were grown in RPMI 1640 supplemented with 5% fetal bovine serum, glutamine and antibiotics (penicillin, streptomycin). The characterization and receptor phenotype for these cell lines has been reported [16, 35].

Survival and growth assays

Cell survival and growth (metabolic activity) was determined by using the colorimetric tetrazolium salt reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) and OD readings were quantified subsequently [20]. Assays were carried out in 96-well microtiter plates (Falcon, Mississauga, Canada) with PC12-wt, PC12-p75^{-} and NNr5-wt plated at 4,000 cells per well and NNr5-TrkC with 6,000 cells per well (pre-determined to be optimal for robust MTT assays, data not shown). Cells were plated in protein-free hybridoma medium supplemented with 0.2% bovine serum albumin (herein referred to as serum free media or SFM), or supplemented with 5% fetal bovine serum. Cells were left untreated and viability was determined at the indicated incubation time points: 24, 48 and 72 h. Assays were repeated at least three independent

times, $n = 3$ to 6 wells per assay. Cell doubling time was determined to be the interval required for a cell population to double in the middle of the logarithmic growth phase [28]. Doubling time values were determined using non-linear regression in GraphPad Prism.

Neurotrophin response assays

Neurotrophin responsiveness of various cell lines was determined using a similar MTT-based protocol. Cells were plated in 96-well plates, and were treated with a range of neurotrophin concentrations from sub-optimal to optimal. Cells were incubated for a total of 48 h. Assays were repeated at least three independent times, $n = 3$ to 6 wells per assay.

Cytotoxicity assays

Cytotoxicity was measured using the MTT-based protocol. Cells were treated with increasing doses of either cisplatin or doxorubicin. Negative and positive controls were untreated cells in normal culture media. IC_{50} values were determined for each cell line via non-linear regression. Then the effect of these pre-determined drug doses were studied in combination with neurotrophin administration (NGF or NT-3). Cell viability was measured after 48 h. Assays were repeated at least three independent times, $n = 3$ to 6 wells per assay.

Soft agar colony formation assay

Soft-agar colony-forming assays were carried out with all four cell lines as a measure of their tumorigenic potential. Cells were plated at 1,000 cells per 100 mm dish (Falcon) in a 0.5% agar layer with 15% FBS. Cells were incubated at 37°C and 5% CO_2 with colonies monitored daily and supplemented with 0.5 ml of 15% FBS weekly. Counting was done at days 18 and 25 after initial inoculation and colonies were classified as large (>50 cells/colony) or small (<50 cells/colony). Mean colony formation were standardized relative to PC12-wt. Experiments ($n = 3$) were performed in triplicate, counting was repeated twice and results were averaged.

In vivo tumor studies

Female CD1 athymic nude mice, seven week old, were injected with single cell suspensions (1×10^6) in a 100 μl volume of saline administered subcutaneously in the left flank. Once each tumor reached a volume of 20–30 mm^3, mice were randomized into three groups with $n = 6$ per group. Group 1 was the control group and received saline treatments. Group 2 was treated with doxorubicin (30 μg per injection). Group 3 was treated with cisplatin (70 μg per injection).

Injections were administered every 3 days for a total of five injections. Drug injections were intraperitoneal on the right flank to ensure systemic drug circulation and to avoid direct contact with the subcutaneous tumors that implanted on the left flank. Tumor volumes were measured using digital calipers, every other day. Tumor volumes were determined using the formula $V = (W^2 \times L)/2$ (W = width, L = length). Once tumor burden exceeded a 15 mm diameter the mice were euthanized and their final tumor weights were determined. All procedures were in accordance with IACUC guidelines.

Statistical analysis

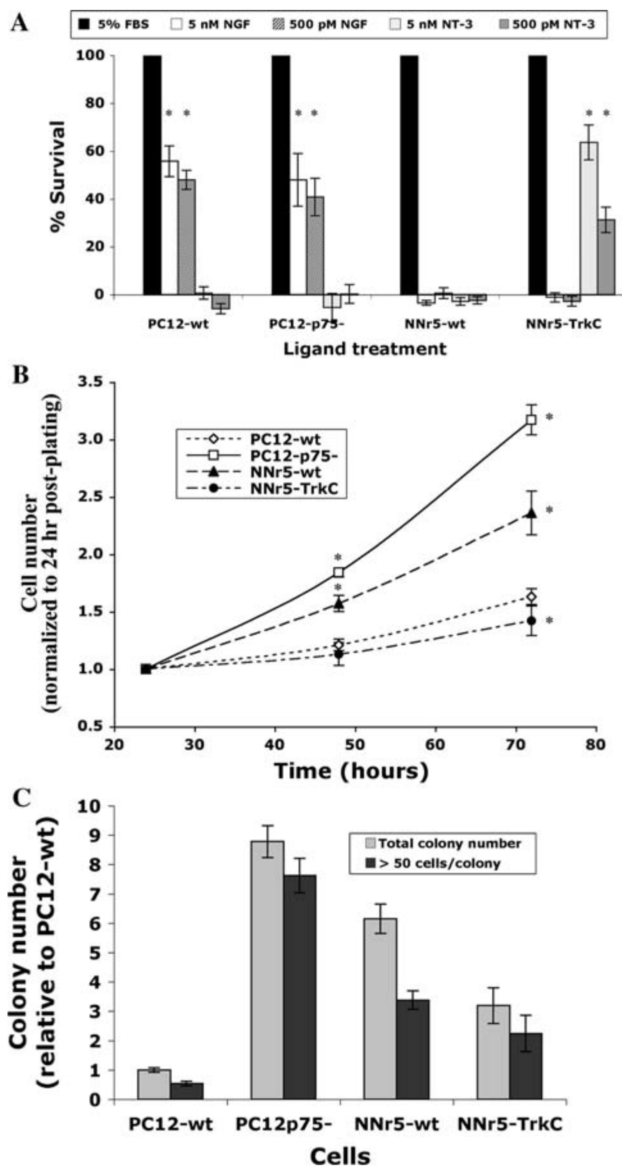
In vitro cytotoxicity assays with doxorubicin and cisplatin and in vivo work was analyzed by non-linear regression followed by independent Student's t tests. Statistical significance for colony-formation assays was analyzed by one-way ANOVA followed by the post hoc Tukey test using GraphPad Prism. $P < 0.05$ was considered significant. All values are represented as means ± standard error of the mean (SEM) of the independent experiments, unless otherwise stated.

Results

Cells expressing TrkA or TrkC and/or p75 have distinct neurotrophin response profiles

The cell surface expression of neurotrophin receptors of rat pheochromocytoma PC12-wt ($TrkA^+$ $p75^{++}$) cells and its variants were determined and routinely monitored by FACScan (data not shown). The variants expressed varying levels of TrkA, TrkC and/or p75 receptors (PC12-$p75^-$: $TrkA^+$ $p75^-$; NNr5-wt: $TrkA^-$ $p75^{++}$; NNr5-TrkC: $TrkC^+$ $p75^{++}$).

The receptors are functional, as assessed by MTT assays. PC12-wt and all its variants undergo apoptosis when grown in serum-free media (SFM), but neurotrophins delay or prevent death if the cells express functional receptors [13]. Cells expressing $TrkA^+$ (PC12-wt, and PC12-$p75^-$), regardless of p75 expression, were protected from death when SFM was supplemented with NGF (5 nM or 500 pM) in a dose-dependent manner, with 5 nM NGF affording optimal protection (Fig. 1a) (as shown previously [13]). Similarly, $TrkC^+$ cells (NNr5-TrkC) were protected from death in SFM supplemented with NT-3 (5 nM and 500 pM). The NT-3 effect was dose-dependent, with the 5 nM dose providing optimal protection. Cells expressing neither TrkA nor TrkC receptors, but p75 alone (NNr5-wt) were not protected from death with either NGF or NT-3.



◀ **Fig. 1** Neurotrophins and receptors afford differential survival, proliferation, and tumorigenesis to PC12-wt and related cell lines. **a** Survival: Cells were cultured in SFM (standardized to 0% survival) supplemented with 5% FBS (growth + survival, standardized to 100%), or in SFM supplemented with the indicated ligands for 48 hours. Cell viability was quantified by MTT. Reported are mean % survival ± standard error of the mean (SEM). Experiments were repeated between five and seven times independently, each in triplicates. $p < 0.05$ was considered significant protection (*). **b** Proliferation: PC12-wt, PC12-p75^{-}, NNr5-wt and NNr5-TrkC were plated in 96-well plates at their optimal growth densities. The proliferation rate was assessed for each cell line. Results are standardized as a fold-difference with respect to PC12-wt growth after 24 h and presented as the mean ± standard error of the mean (SEM). Experiments were repeated four independent times, each in triplicate. Data was analyzed by one-way ANOVA followed by post hoc Tukey's test. *indicates significant difference at 48 h; and at 72 h all cells were significantly different from PC12-wt cells, $p < 0.05$. **c** Colony formation. Soft agar colony formation assay of PC12-wt, PC12p75^{-}, NNr5-wt and NNr5-TrkC. Colonies were classified as small (less than 50 cells per colony) or large (more than 50 cells per colony), counted twice and then averaged both at day 18 (data not shown) and day 25. Results obtained for cell variants were standardized to PC12-wt colony count results. Results from colony formation assays were analyzed by one-way ANOVA followed by the post hoc Tukey test. Total colony counts and colonies with more than 50 cells per colony are shown. Experiments were repeated at least three independent times. All values are represented as means ± standard error of the mean (SEM), $p < 0.05$ considered significant. All cell lines formed significantly higher number of colonies, of larger size, than PC12-wt cells

These results are consistent with the expression of receptors mediating the expected functional signals in response to ligands.

Cellular proliferation rate depends on the expression profile of Trk and p75 receptors

To assess whether differences exist in the growth kinetics of the various cell lines, proliferation of cells was studied by culturing cells in optimal growth medium (5% FBS) using the MTT assay (Fig. 1b). Doubling time was determined to be the interval required for a cell population to double in the middle of the logarithmic growth phase [28]. Similar data were also obtained with ^{3}H-thymidine incorporation assays as a measure of DNA replication (data not shown).

Initial experiments monitored growth at several initial plating cell densities for each cell line, to ascertain optimal conditions for measuring proliferation rates. These plating densities are 4,000 cells per well for PC12-wt, PC12-p75^{-} and NNr5-wt; and 6,000 cells per well for NNr5-TrkC (data not shown). Proliferation assays showed marked differences in doubling time over a time span of 72 h, with cells expressing only TrkA or p75 having the fastest growth rates. Cells expressing TrkA had the highest proliferation rate (PC12-p75^{-} ~29 h), with p75 expressing cells also proliferating at significantly high rates (NNr5-wt ~39 h). In contrast, cells co-expressing either TrkA or TrkC with p75 had significantly slower proliferation rates. Cells expressing TrkA with p75 (PC12-wt ~66 h) had a significantly faster doubling time than TrkC cells co-expressed with p75 (NNr5-TrkC ~92 h) which had the slowest growth kinetics.

In vitro tumorigenic potential depends on neurotrophin receptor expression

Following the characterization of PC12-wt and its variants, we wished to determine if differences in neurotrophin receptor expression affect tumorigenic potential. Cells were thus tested for their ability to form colonies in agar (Fig. 1c). Different receptor expression (TrkA, TrkC and/or p75) in PC12-wt and its variants led to significant differences in the tumorigenic potentials of these cells.

Results were standardized to the colonies formed by PC12-wt for ease of comparison. PC12-wt had a total

number of colonies 87 ± 7; out of which 47 ± 3 (~50%) were large colonies with more than 50 cells each. Colony data are reported for day 25 after initial seeding but counting at day 18 was also done. Data for day 18 are not shown because both the relative number of colonies and the proportion of large colonies are similar to the data for day 25.

Cells expressing TrkA or p75 alone had significantly higher colony-forming potentials than cells co-expressing TrkA or TrkC with p75. PC12-p75⁻ (expressing only TrkA) had the highest colony-forming potential, close to ninefold more than PC12-wt cells. Moreover, PC12-p75⁻ cells developed the highest proportion of large colonies, ~90% of all colonies had more than 50 cells per colony. The NNr5 cells (expressing only p75) had a high colony-forming potential, close to sixfold higher than PC12-wt cells, and ~50% of all colonies had more than 50 cells per colony.

Interestingly, NNr5-TrkC cells (expressing TrkC and p75) had a higher colony-forming potential than PC12-wt cells (expressing TrkA and p75), and ~70% of all colonies had more than 50 cells per colony. Thus, PC12-wt cells developed the smallest number of colonies and the lowest proportion of large colonies.

It is noteworthy that these colony-forming data (Fig. 1c) contrasts to the proliferation studies (Fig. 1b) showing that PC12-wt cells in liquid culture doubled significantly faster than the nnr5-TrkC cells.

Differential neurotrophin receptor expression confers unique cellular sensitivity profiles to doxorubicin and cisplatin

Next, we evaluated if variations in neurotrophin receptor expression provide cells with different sensitivities to chemotherapeutic agents. Drug concentration–response profiles of PC12-wt and its variants were studied by MTT. Cells were cultured in complete media ± increasing doses of doxorubicin or cisplatin. Results show that cells have significantly different IC_{50} values to chemotherapeutic treatments (Table 1; Fig. 2). Similar data were also obtained with ^{3}H-thymidine incorporation assays as a measure of DNA replication (data not shown).

Table 1 IC_{50} values of Doxorubicin and Cisplatin for PC12-wt and its variants

Cell type	Doxorubicin IC_{50} (μM)	Cisplatin IC_{50} (μM)
PC12-wt	0.943 ± 1	5 ± 2
PC12-p75⁻	0.610 ± 1	32 ± 1
NNr5-wt	0.686 ± 1	15 ± 1
NNr5-TrkC	0.891 ± 1	44 ± 2

Cell survival was tested by MTT assay for all cell lines after culture in serum and increasing doses of doxorubicin or cisplatin for 48 h. IC_{50} values were determined by non-linear regression analysis using GraphPad Prism

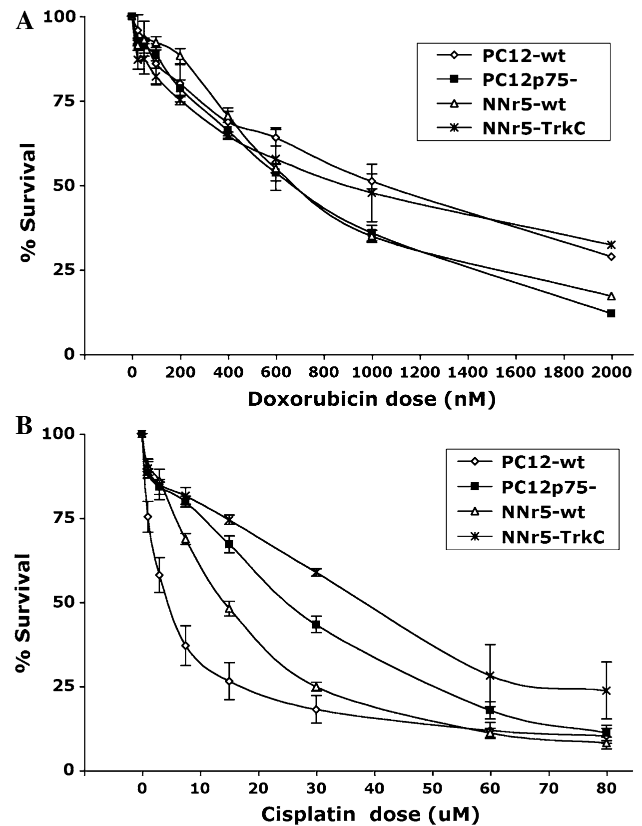


Fig. 2 Concentration–response curves to doxorubicin or cisplatin. Cells were treated with increasing drug doses for 48 h. **a** Doxorubicin (nM), **b** cisplatin (μM). Cytotoxicity was quantified via the MTT assay. Cell survival was standardized to untreated cells (no drug, 100%) under optimal growth conditions. % survival ± SEM was determined from three to five independent experiments, each in triplicates, with $p < 0.05$ considered significant. Table 1 summarizes IC_{50}

In general, all the cell lines were more sensitive to doxorubicin (nM range) than to cisplatin (μM range) (Table 1). NNr5-TrkC cells were the most resistant to cisplatin (IC_{50} = 44 μM); ~ninefold higher than PC12-wt, which was the cell line most sensitive to cisplatin (Fig. 2b). The sensitivity of the various cell lines to doxorubicin took place over a narrower range. PC12-wt were the most resistant to doxorubicin (IC_{50} = 943 nM); ~1.5-fold higher than PC12-p75⁻, which was the cell line most sensitive to doxorubicin (Fig. 2a) (Table 2).

Administration of neurotrophins rescues Trk-expressing cell lines differently from doxorubicin or from cisplatin cytotoxicity

To determine the effect of neurotrophins on the cytotoxic effects of doxorubicin or cisplatin, PC12-wt and its variants were treated with either doxorubicin (Fig. 3a) or cisplatin (Fig. 3b) in complete media ± optimal concentrations (5 nM) of NGF or NT-3.

Table 2 Summary of data presented

Cell line	In vitro doubling time (h)	Total colony # in agar	Drug sensitivity IC_{50}		% Cell survival Cytotoxic drugs ± NTF (% of maximal, drug untreated cells)					
			Doxo	Cisp	Doxo	Doxo + NGF	Doxo + NT-3	Cisp	Cisp + NGF	Cisp + NT-3
PC12-wt	~66	87 ± 7	0.943 ± 1	5 ± 2	34 ± 5	93 ± 5	44 ± 10	52 ± 2	103 ± 5	55 ± 4
PC12-p75⁻	~29	767 ± 51	0.610 ± 1	32 ± 1	49 ± 7	72 ± 10	59 ± 12	54 ± 9	49 ± 13	60 ± 22
NNr5-wt	~39	538 ± 27	0.686 ± 1	15 ± 1	39 ± 9	57 ± 17	53 ± 14	47 ± 6	52 ± 6	50 ± 21
NNr5-TrkC	~92	279 ± 54	0.891 ± 1	44 ± 2	77 ± 2	78 ± 3	105 ± 8	20 ± 1	25 ± 4	24 ± 3

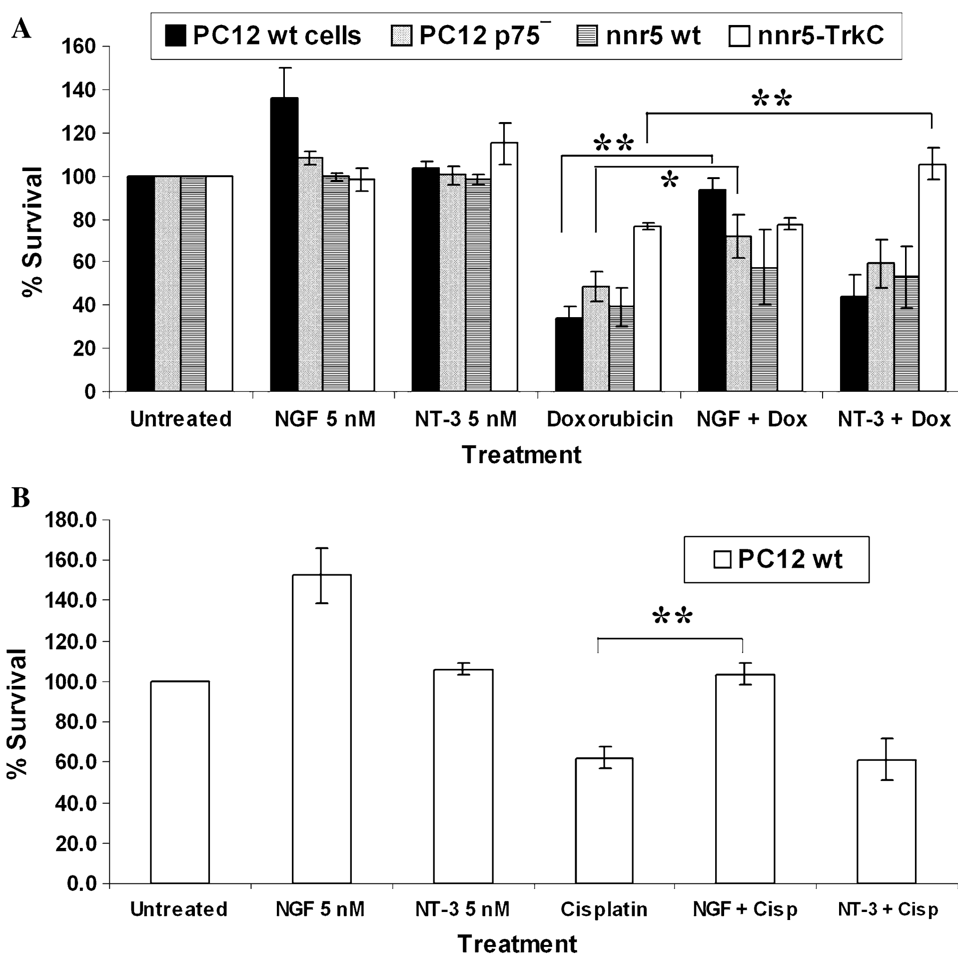


Fig. 3 Neurotrophins rescue cells differentially from doxorubicin or cisplatin-induced apoptosis. Cells were cultured in serum-containing media for 48 h, and their metabolism (growth + survival) was analyzed by MTT assays. Similar data were also obtained with ^{3}H-thymidine incorporation assays as a measure of DNA replication (data not shown). Each cell line was treated with either doxorubicin or cisplatin at their approximate IC_{50}, and 5 nM NGF or NT3. **a** Responses of all cells to doxorubicin ± neurotrophins. **b** PC12-wt treated with cisplatin ± neurotrophins. The other cells lines (PC12-p75⁻, NNr5-wt, NNr5-TrkC) are not shown because they were not protected at all from cisplatin toxicity by the neurotrophins. Assays were repeated three independent times, each in triplicates. *$p < 0.05$; **$p < 0.01$. Data are the mean of three assays ± SEM

For doxorubicin-treated cells, NGF rescued PC12-wt cells (doxorubicin + NGF 93 ± 5% survival vs. doxorubicin 34 ± 5%), and PC12-p75⁻ to a lesser significance (doxorubicin + NGF 71 ± 10% survival vs. doxorubicin 49 ± 9%). For cisplatin-treated cells, however, NGF rescued only PC12-wt cells (cisplatin + NGF 103 ± 5% survival vs. cisplatin 52 ± 2%) (Fig. 3b); and NT-3 did not protect PC12-wt cells from cisplatin cytotoxicity. Thus, TrkA agonism by NGF has the ability to protect some cells from some types of chemotherapeutic damage.

Neither the PC12-wt cells nor the PC12-p75⁻ cells were rescued by NT-3 from doxorubicin cytotoxicity (Fig. 3a) or from cisplatin toxicity (data not shown), although this neurotrophin be a TrkA agonist [14, 36]. These data indicate that TrkA agonism by NT-3 and NGF also differ with respect to protection of cytotoxicity; consistent with reports that these ligands differ in terms of their TrkA binding affinity and activation of signaling pathways [13, 14].

For doxorubicin-treated cells, NT-3 rescued NNr5-TrkC (doxorubicin + NT-3 105 ± 8% survival vs. doxorubicin 77 ± 2%). However, for cisplatin-treated cells there was no protection afforded by NT-3 to any cell line, including NNr5-TrkC (data not shown). These data indicate that TrkC agonism can protect from some types of chemotherapeutic damage.

No protective effects were observed by administration of NGF to NNr5-TrkC cells treated with doxorubicin (Fig. 3a) or with cisplatin (data no shown). This was expected because NGF does not activate TrkC. However, the data also indicates that a p75 ligand (e.g. NGF acts as a p75 ligand in this paradigm) has no effect. Also as expected, no protective effects were observed by administration of NGF or NT-3 to NNr5-wt cells treated with doxorubicin (Fig. 3a) or with cisplatin (data no shown).

Together, these data indicate that in these cells specific ligand activation of Trks can afford protection from some types of cytotoxic death (e.g. doxorubicin) but not others (e.g. cisplatin). It also appears that ligand-binding to p75 does not affect chemotherapeutic cytotoxicity. Thus, our data suggest that neurotrophins may have a selective protective effect towards doxorubicin cytotoxicity and not towards cisplatin cytotoxicity.

It is noteworthy that the effects of neurotrophins in these assays include not only protection form cytotoxicity but also induction of differentiation. However, it is unlikely that the effects observed are due to cellular differentiation induced by neurotrophins. First, the time course of these experiments (<48 h) does not allow for efficient differentiation in these cell lines (>96 h). Second, PC12-p75$^-$ cells are not protected by NGF although they can differentiate in response to this neurotrophin. Third, neurotrophins do not protect cells from both chemotherapeutic agents even though they both kill with similar mechanisms (by intercalating or binding to DNA and interfering with repair mechanisms and topoisomerases).

In vivo tumor growth kinetics

With our in vitro studies showing that significant differences exist between the growth kinetics and tumorigenic potential of the various cell lines studied, we then wished to evaluate tumorigenicity in vivo. Nude mice were implanted subcutaneously on their flank with the four cell lines and once tumors reached a volume of 20–30 mm^3, animals were randomized into three groups ($n = 6$ per group). Mice were treated with saline, doxorubicin or cisplatin, for a total of five injections at 3-day intervals. Measurements were started on day 0 which corresponds to the randomization of the animals and the first day of treatment administration. Results show that significant differences exist in the in vivo growth kinetics of PC12-wt and its variants (Fig. 4a).

PC12-wt bearing mice develop the most aggressive tumors in terms of volume (~1,600 mm^3) and doubling time (~4.4 days). This is consistent with in vitro doubling times (Fig. 1b), but is somewhat surprising because of the lower transforming potential of PC12-wt cells in colony forming assays (Fig. 1c).

PC12-p75$^-$ cells also develop large tumors in mice (~780 mm^3) with similar proliferation rates as PC12-wt cells (~4.4 days).

NNr5-TrkC cells have very weak tumor bearing potential in mice, and only grew in 1/3 of the injected animals. Those tumors that did grow doubled at about 13 days and resulted in small volumes (~164 mm^3) even after 50 days.

NNr5-wt cells also did not develop any significant tumor burden in mice. The in vivo growth rate of NNr5-wt cells is significantly slower than PC12-p75$^-$ cells, with tumors doubling every 6 days and remaining very small (~158 mm^3) even after 50 days.

In vivo tumor responsiveness to doxorubicin and cisplatin

The effect of doxorubicin and cisplatin administration on tumor growth in vivo could only be evaluated in PC12-wt and PC12-p75$^-$ cells because NNr5-wt and NNr5-TrkC cells did not develop tumors in a sufficient number of animals to achieve statistical validity.

The growth of PC12-wt tumors was not affected by doxorubicin. However, PC12-wt tumors were sensitive to cisplatin treatment and tumor burden was significantly lower than the saline control group ($p < 0.05$ at days 15 and 17 of the experiment, Fig. 4b).

The growth of PC12-p75$^-$ tumors was not affected by either doxorubicin or cisplatin (data not shown), and tumors in drug-treated animals were indistinguishable from saline controls.

Discussion

Trk activities have been described in a variety of cancers of both neuronal and non-neuronal origin. Neurotrophic action is involved in the regulation of tumor survival, proliferation and differentiation. Additionally, the role of p75 in cancer has only recently started to be elucidated in a limited number of tumor models and in human biopsies.

In the present study, we characterized the growth kinetics and tumorigenic potential of native PC12-wt cells and derived daughter cell lines expressing TrkA or p75 alone or co-expressed with TrkA or TrkC. Subsequently, we quantified the biological response of these cells in response to chemotherapy and ligand administration. These studies provide information useful for understanding the possible roles of p75 and Trks in tumorigenesis as well as how they interplay with each other.

It is recognized that systematic differences between growth rates exist between tumors of different histological types. In order to control such variation, we selected a cellular model based on a common origin and then derived related cell lines for our studies. The PC12 cell model,

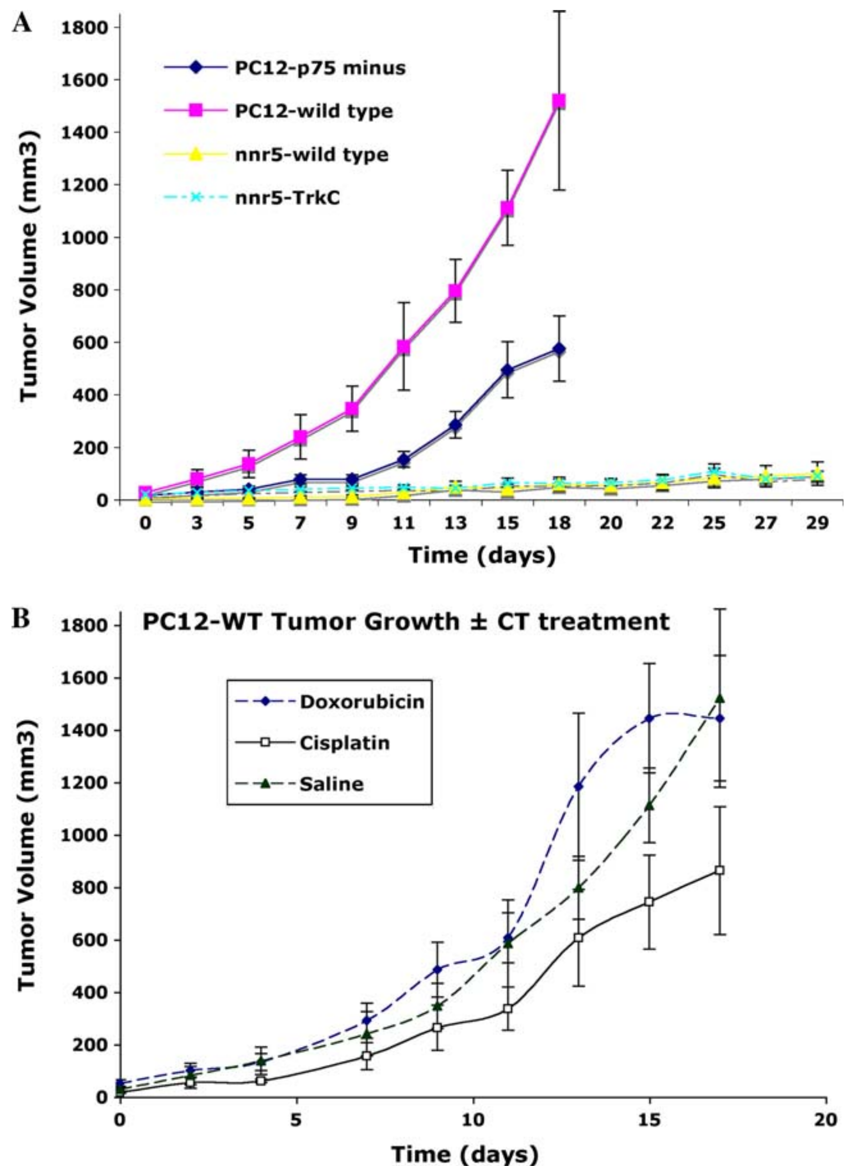


Fig. 4 In vivo tumor growth kinetics and chemosensitivity. Nude mice were implanted subcutaneously on the flank with 1×10^6 cells, as a single cell suspension. **a** Growth kinetics of the control groups for the four tumor types. NNr5-wt and NNr5-TrkC tumor progression was monitored for an additional 20 days, with no significant increase in tumor volumes. **b** PC12-wt tumors were administered three treatments: saline control, doxorubicin 30 µg, or cisplatin 70 µg (both below the lethal toxic dose) initiated when tumors reached a volume of 20–30 mm^3. Treatments consisted of a total of five injections, once every 3 days, with the first injection at day 0. Tumor volume was measured at the indicated intervals and calculated as per: $V = (W^2 \times L)/2$ (W = width, L = length). Cisplatin reached significance, $p < 0.05$ versus saline, at days 15 and 17

derived from rat pheochromocytoma, a tumor of the adrenal medulla or sympathetic ganglia, has been extensively used to study the signaling pathways leading to neuronal survival and differentiation regulated by neurotrophins (NGF) and growth factors (EGF) [33]. However, even within tumors of similar histological origin, growth rates can differ significantly [19]. This is often due to phenotypic tumor traits that can affect invasiveness and metastatic potential.

Accordingly, our data demonstrates that significant doubling time differences exist between variants of the PC12-wt cells used in our model. Previous studies have shown PC12-wt doubling time to be slow (~3 days) [3]. Our data is consistent with this finding (our PC12-wt doubling time ~66 h). While we have not seen any other alterations to these cell lines, unknown phenotypic changes may result as consequence of constitutive alterations to neurotrophin receptors. For that reason, it will be important to expand these studies to other cell lines or to primary tumors.

What might account for the differential growth patterns of these cells in a model with no exogenous neurotrophin? Reports have documented that p75 and Trks can cross-regulate each other's function, even with no neurotrophins [20]; and that TrkA-p75 and TrkC-p75 regulation differs and leads to distinct biological outcomes [13]. This may explain the differences we detect between TrkA or TrkC-induced tumorigenicity with and without p75 expression. Also, expression of accessory molecules such as Galectin-1 [5], or Sall2 (a p75-interactor) [26] can affect the phenotype in a ligand-independent manner. However, our data argues against a role for ligand-dependent roles for p75, as NGF as a p75 ligand did not affect tumor growth or chemosensitivity in Nnr5-TrkC, and no ligand affected Nnr5-wt cells either. Other reports have shown that autocrine pathways

can in part account for chemoresistance wherein the tumor secretes a neurotrophin [12]; or expresses an activated form of the receptor [18].

Our results are consistent with but expand on the previous reports. We find that TrkA co-expressed with p75 in PC12-wt cells leads to a more favorable tumor profile that responds to cisplatin therapy. However, when TrkA is expressed alone, PC12-p75$^-$ tumors double quickly, reach large volumes and present resistance to both doxorubicin and cisplatin in vivo. This is consistent with reports linking p75 expression to cellular sensitivity to oxidative stress [16, 35]. Also, TrkA has been found to trigger apoptosis [17], or cell cycle arrest and differentiation [21, 24] in neuroblastoma. Indeed, TrkA expression in neuroblastomas correlates with low tumor grade and favorable outcomes, whereas downregulation of the TrkA receptor is linked to a more aggressive tumor phenotype [23].

High TrkC expression correlates with favorable outcomes for medulloblastoma patients, whereas low TrkC levels correlate with higher risk of death. Thus TrkC is a predictor of clinical outcome [9, 29]. Our data shows that cells expressing TrkC with p75 have low tumorigenic potential and slow growth kinetics. However, the fact that these cells are resistant to both doxorubicin and cisplatin in vitro and are rescued by NT-3 from chemotherapeutic cytotoxicity suggests that treatment of these tumors may be challenging.

Low in vivo tumorigenic potential of p75 expressing cells correlates well with studies that show that p75 expression induces significant increase in apoptosis of muscular sarcoma [27]. Similarly, p75 expression increases apoptosis of prostate cancer cells [8], while TrkA and TrkB expression and signaling enhances cellular malignancy. On the other hand, glioblastomas appear to express p75 at the invasive/migrating edge [15]. Unfortunately this observation has not been followed to show neurotrophin responses or a role for chemotherapy sensitivity or resistance.

Treatment with tyrosine kinase inhibitors or by anti-NGF antibodies that block TrkA activity decreases cell proliferation [27]. Similarly, BDNF protects neuroblastoma cells from treatment with cisplatin and doxorubicin, suggesting that it alters a common signaling pathway required for cell death initiation by DNA damaging agents [22]. This correlates with our data indicating that in vitro, treatment of cells with neurotrophins in combination with cisplatin or doxorubicin increases cell survival and rescues most cell lines from cell death. This opens the door for potential targeted therapy of tumors expressing neurotrophin receptors with antagonists to enhance the efficacy of conventional therapy.

The differential in vitro sensitivity and IC_{50} profiles to doxorubicin (and to some extent to cisplatin) do not simply reflect differences in proliferation rates of the four cell lines. For example, in the case of cisplatin, pairs of cell lines with "similar" doubling times (i.e., PC12-wt and Nnr5-TrkC; Nnr5-wt and PC12-p75$^-$) do not have similar IC_{50} profiles. This is relevant because both chemotherapeutic agents are known to kill in a cell-cycle dependent mechanism of action, by intercalating or binding to DNA and interfering with repair mechanisms and topoisomerases, leading to cell death.

Overall these data suggest that proliferation rate alone does not account the differential sensitivity of these cell lines to different agents. One interpretation is that the differential expression of neurotrophin receptors accounts for at least some of the differential sensitivity.

This interpretation may also account for whether or not neurotrophins can protect from cell death induced by each chemotherapeutic agent. NGF can protect TrkA-expressing cells (i.e., PC12-wt and PC12-p75$^-$) from doxorubicin but only protects PC12-wt from cisplatin. NT-3 can protect TrkC-expressing cells (i.e., Nnr5-TrkC) from doxorubicin but not from cisplatin.

Our results show that precautions must be taken when interpreting in vitro data for clinical significance for the entire organism, as significant discrepancies can occur between *ex vivo* and in vivo studies. This emphasizes the fact that tumor growth, although well controlled in the laboratory setting, is affected by the contact of intrinsic cellular factors, the extracellular matrix, stromal cells and other host factors [34]. Skin keratinocytes produce NGF [25, 30], and this may explain why TrkA expressing cells were more tumorigenic in vivo, as local neurotrophin production can stimulate growth. Furthermore, it has been shown that co-expression of Trk with p75 enhances high affinity binding with neurotrophins and promotes survival [7, 32]. This can explain why TrkA-p75 cells (PC12-wt) proliferate more significantly in vivo in contrast to TrkA only expressing cells (PC12-p75$^-$). As the role of p75 in NGF signaling is still unclear, this evidence can suggest that, in skin, p75 enhances the proliferation and survival of tumor cells when co-expressed with TrkA.

These results have implications that warrant additional evaluation for developing tailored therapies.

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