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p75NTR: an enhancer of fenretinide toxicity in neuroblastoma

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Abstract

Objective—Neuroblastoma is a common, frequently fatal, neural crest tumor of childhood. Chemotherapy-resistant neuroblastoma cells typically have Schwann cell-like (“Stype”) morphology and express the p75 neurotrophin receptor (p75NTR). p75NTR has been previously shown to modulate the redox state of neural crest tumor cells. We, therefore, hypothesized that p75NTR expression level would influence the effects of the redox-active chemotherapeutic drug fenretinide on neuroblastoma cells.

Methods—Transfection and lentiviral transduction were used to manipulate p75NTR expression in these cell lines. Sensitivity to fenretinide was determined by concentration- and time-cell survival studies. Apoptosis incidence was determined by morphological assessment and examination of cleavage of poly-ADP ribose polymerase and caspase-3. Generation and subcellular localization of reactive oxygen species were quantified using species- and site-specific stains and by examining the effects of site-selective antioxidants on cell survival after fenretinide treatment. Studies of mitochondrial electron transport employed specific inhibitors of individual proteins in the electron transport chain.

Results—Knockdown of p75NTR attenuates fenretinide-induced accumulation of mitochondrial superoxide and apoptosis. Overexpression of p75NTR has the opposite effects. Pretreatment of cells with 2-thenoyltrifluoroacetone or dehydroascorbic acid uniquely prevents mitochondrial superoxide accumulation and cell death after fenretinide treatment, indicating that mitochondrial complex II is the likely site of fenretinide-induced superoxide generation and p75NTR-induced potentiation of these phenomena.

Conclusion—Modification of expression of p75NTR in a particular neuroblastoma cell line modifies its susceptibility to fenretinide. Enhancers of p75NTR expression or signaling could be potential drugs for use as adjuncts to chemotherapy of neural tumors.

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Conflict of interest

None of the authors have any real or perceivable conflicts of interest or financial relationships relevant to the contents of this manuscript.

Keywords

Neuroblastoma; Neurotrophin receptor; Mitochondria; Oxidative stress; Retinoid

Introduction

The p75 neurotrophin receptor (p75NTR) is expressed throughout the neuraxis during embryogenesis. Its expression is increasingly restricted during development and quite anatomically limited in adulthood [1]. It has been used as one marker of “stemness” in the nervous system [2] and persists in developmental neoplasia of the peripheral and central nervous systems [3–5]. We and others have demonstrated the variably pro- and anti-apoptotic effects of signaling through p75NTR [4, 6–8], and our prior studies suggest that p75NTR signaling influences the redox state of the cell [9, 10].

Neuroblastoma, a developmental neoplasm of the neural crest [11], exhibits variable expression of p75NTR [12]. The most chemoresistant neuroblastoma cells, the S- or Schwann-type cells, exhibit high expression of p75NTR and constitute residual disease after therapy for disseminated neuroblastoma [13, 14]. Children with large, bulky primary tumors or metastatic disease at the time of diagnosis constitute 65 % of children with neuroblastoma and have a 20–50 % chance of surviving 5 years after diagnosis [11].

One of the most interesting aspects of neuroblastoma is its ability under certain circumstances to differentiate into post-mitotic cells and to undergo apoptosis. This ability has fueled efforts to mimic this differentiation using chemotherapeutic drugs. Retinoic acid is one differentiating agent studied in patients with neuroblastoma. Although in vitro studies were encouraging, in vivo results were complicated by the development of resistance to the drug [15].

Fenretinide (4-hydroxyphenyl retinamide) was developed as a retinoic acid analogue in an attempt to improve on the pharmacokinetics of 13-*cis*-retinoic acid and avoid the development of resistance. However, fenretinide did not bind avidly to retinoic acid receptors and induced apoptosis, rather than differentiation, of neuroblastoma cells [16, 17]. A recent study of fenretinide-induced apoptosis demonstrated mitochondrial accumulation of reactive oxygen species (ROS) [18].

In view of our prior demonstration of the redox activity of p75NTR signaling [9, 10] and the putative oxidative effects of fenretinide [18], we sought to determine the effects of p75NTR expression on susceptibility to fenretinide-induced mitochondrial accumulation of ROS and apoptosis.

Materials and methods

Chemicals and reagents

Fenretinide, Hoechst dye #33342, dehydroascorbic acid (DHA), N-acetylcysteine, 2-thenoyltrifluoroacetone (TTFA), and antimycin A were purchased from Sigma Chemical Corp. (St. Louis, MO, USA). MitoSOX™ Red mitochondrial superoxide indicator, dihydroethidium(DHE), and 5- (and 6-) carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) were obtained from Molecular Probes, Inc. (Eugene, OR, USA). Alamar blue and trypan blue were purchased from Invitrogen (Carlsbad, CA, USA). MTS assay reagent (CellTiter 96 Aqueous One Solution) was obtained from Promega (Madison, WI).

Cell lines and cell culture

Human neuroblastoma S-type cell lines, SH-EP1 and SK-N-AS, and the N-type cell line, SH-SY5Y, were purchased from American Type Culture Collection (ATCC, Rockville, MD) and maintained as recommended by the ATCC. Cells were plated 24–48 h prior to transfection or lentiviral transduction, and fenretinide treatment occurred 24 h after transfection or 48 h after transduction.

Knockdown of p75NTR

p75shRNA, a mixture of three different shRNA sequences, was purchased from Santa Cruz Biotechnologies and transfected using an Amaxa Nucleofector II. For example, SH-EP1 cells were transfected with Nucleofector Kit V as recommended by the manufacturer. Briefly, cells were plated at 70 % confluency 24–48 h prior to transfection; 2×10^6 cells were trypsinized and the supernatant was centrifuged and removed. The pellet was resuspended in a 100 ml mixture of nucleofector solution (82 %) and supplement solution (18 %) and added into the cuvette followed by nucleofection using the corresponding program in the Nucleofector II machine (Amaxa). The transfected cells were plated in 6-cm dishes and treated with the resistance antibiotic (5 µg/ml puromycin) to create stably transfected cells.

Transient knockdown of p75NTR was also performed by lentiviral transduction using each of four different p75NTR/scrambled shRNA pairs in order to be certain that the effects seen were not unique to a particular shRNA construct or a particular component of the p75NTR molecule. In addition, these transductions were performed in SH-EP1, SK-N-AS, and SH-SY5Y cells to ensure that the effects of knockdown were not unique to SH-EP1 cells. The forward and reverse primers were reconstituted and annealed using annealing buffer and by running the PCR at 95 °C for 4 min. The annealed primers were allowed to cool at room temperature for 10 min and then placed on ice. The annealed oligonucleotides were diluted with 140-µl water and the ligation reaction was performed with the oligonucleotides present and with appropriate controls (i.e., without oligonucleotides and without ligase, respectively) using T4 ligase, buffer, and the pLKO.1 digested vector (AgeI/EcoRI); the PCR was run at 16 °C overnight. The competent cells were transformed with 10 µl of the ligation reaction on ice followed by a 42 s heat shock and replacement on ice. The competent cells were incubated in super optimal broth with catabolite repression medium, shaking at 225 rpm and 37 °C for 1 h. An aliquot (300–400 µl) of the culture was streaked onto a lysogeny broth (LB) plate containing ampicillin and grown overnight. Three colonies were picked for each construct and grown in a glass tube with LB. Three ml of the bacterial culture was centrifuged and the DNA was isolated using the Qiagen Miniprep kit (Qiagen, Valencia, CA, USA). The presence of the insert was detected by running restriction enzyme digests on an agarose gel and the positive clones were sequenced. The positive clones were packaged into virus grown in 293TN cells which were used to transduce the neuroblastoma cells with p75shRNA. Following a 6-h infection, the cells were allowed to grow in medium and trypsinized 24-h post-infection for qPCR, Western blotting, and drug treatments. The cells were agitated using Buffer RLT (Qiagen) with β-mercaptoethanol, and the RNA was extracted from the cells using an RNeasy isolation kit (Qiagen). Genomic DNA was removed and the RNA was eluted for concentration analysis using a NanoDrop (Invitrogen, Grand Island, NY). RNA (500–1,000 ng) was reverse-transcribed into cDNA in a thermocycler and used for qPCR. The qPCR was performed using the p75NGFR primer probe which was normalized to a GAPDH control. The two transductants with the lowest p75NTR expression (transductants 5-1 and 6-1; shRNA sequences GGAACAGCTGCAAGCCCTACA and GCACTGTAGTAAATGGCAATT, respectively) were used for further studies.

p75NTR-overexpressing SH-EP1 cells

Transfection of SH-EP1 cells with a constitutive expression construct for full-length p75NTR was performed and verified as described previously by our laboratory [9, 10].

Fenretinide treatment and assays of cell membrane integrity, metabolic viability, survival, and apoptosis

Cells were treated for 24–72 h at 37 °C in complete medium with fenretinide (0–20 µM). Subsequent to treatment, cells were assayed for membrane integrity (trypan blue, lactate dehydrogenase (LDH) release), metabolic viability (Alamar blue, MTS), survival (adherent cell counts), and apoptosis (poly-ADP ribose polymerase (PARP) and caspase-3 cleavage, Hoechst dye-stained nuclear morphology) using standard, previously described techniques [19–21].

Western blotting

Native, p75NTR-deficient, and p75NTR-overexpressing neuroblastoma cells were lysed in radioimmunoprecipitation buffer (10 mM Tris, pH 8, 150 mM NaCl, 0.1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 mM PMSF, 4 mg/ml aprotinin, 1 mM sodium orthovanadate) for at least 30 min on ice. Subsequently, the protein concentration was estimated in triplicate samples using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as a standard. Equivalent amounts of protein-containing lysates were loaded onto each lane and electrophoresed in 7.5 % SDS–polyacrylamide gel, followed by blotting onto a nitrocellulose membrane (Bio-Rad Laboratories). After blotting, non-specific binding was blocked with 5 % non-fat dry milk in PBS for 1 h and the membrane was incubated for 2 h at room temperature with primary antibodies (anti-p75NTR antibody, 1:1,000; Promega, Madison, WI) diluted into 5 % non-fat dry milk in PBS. The membrane was then washed with PBS and incubated with secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:1,000; Santa Cruz Biotechnology) for 1 h. The membrane was finally washed and developed with Western blotting chemiluminescence luminol reagent (Santa Cruz Biotechnology) following the manufacturer's instructions. The procedure was repeated staining for actin as a loading standard. Densitometric analysis was performed using Scion Image, and the data were presented as the average ratio of p75NTR/actin from at least three independent experiments.

Assays of ROS accumulation

Cellular concentrations of peroxide were determined using carboxy-H2DCFDA; cellular concentrations of superoxide were determined using DHE; and mitochondrial generation of superoxide was determined using the mitochondrial superoxide indicator MitoSOX™ reagent.

For each reagent, neuroblastoma cells growing in 96-well plates were stained following the manufacturers' instructions. Briefly, cells were washed once with warm HBSS/Ca/Mg and incubated in 2 µM carboxy-H2DCFDA, 5 µM DHE, or 2 µM MitoSOX™ Red for 30 min at 37 °C (protected from light) followed by three washes with warm buffer. The fluorescence was then measured with a fluorescence plate reader (SpectraMax M5) at the excitation wavelength of 488 nm for carboxy-H2DCFDA and 505 nm for DHE and MitoSox™. An emission wavelength of 535 nm was used for carboxy-H2DCFDA and 610 nm was used for DHE and MitoSox™. For quantifications, sister cultures treated identically were incubated with Alamar blue reagent according to manufacturer's instructions. Carboxy-H2DCFDA and DHE fluorescence were corrected for the number of viable cells as determined by Alamar blue assay. In addition, cells were also viewed under a Nikon TE 2,000 light microscope equipped for epifluorescence illumination to monitor the intracellular

distribution of the fluorescence. Since all of the dyes used are light sensitive, low light conditions were maintained whenever possible. A SPOT digital camera with manual control was used to ensure the same exposure time for all pictures. Where noted in the figure legends, relative carboxy-H2DCFDA staining was alternatively quantified by spectrophotometry at a wavelength of 485 nm as specified by the manufacturer.

Antioxidant and mitochondrial complex inhibitor treatment

DHA (400 μ M) was used to scavenge mitochondrial ROS. N-acetylcysteine (5 mM) was used to enhance the production of predominantly cytoplasmic glutathione and, thereby, to reduce ROS selectively in that compartment. Pretreatment with DHA or N-acetylcysteine for 24 h was followed by fenretinide treatment (72 h; 0–20 μ M). Rotenone (0–80 μ M) was used to inhibit complex I; TTFA (0–2 mM) to inhibit complex II; and antimycin A (0–40 μ M) to block complex III by pretreating the cells for 4 h prior to fenretinide treatment (24 h; 0–20 μ M).

Results

S-type neuroblastoma cells are susceptible to fenretinide-induced apoptosis

Fenretinide treatment (24–72 h; 0–20 μ M) reduces cell viability in all human neuroblastoma cell lines on which it was tested, including two S-type (SH-EP1 and SK-N-AS) and one N-type (SH-SY5Y) lines (data not shown). For example, in SH-EP1 cells, the effects of fenretinide on cell survival, as assessed by cell counts, trypan blue exclusion, lactate dehydrogenase release, and MTS and Alamar blue staining, are both concentration- and time-dependent (Fig. 1a–d). As phase I clinical trials of fenretinide in neuroblastoma demonstrated that the maximum tolerated dose resulted in a plasma concentration of 10 μ M [22], these in vitro studies were performed to ensure that significant neuroblastoma cytotoxicity occurs at and below this concentration. The LC₅₀ for fenretinide (72 h exposure) is just above that concentration at 14 ± 0.2 μ M and 12 ± 1 μ M for SH-EP1 and SK-N-AS cells, respectively. Concentration- and time-dependent fractional cleavage of PARP and caspase-3 (Fig. 2a–c) and nuclear condensation and fragmentation (Fig. 2d, e) are seen as well, confirming that fenretinide-induced cell death is apoptotic in nature.

Modulation of p75NTR receptor expression alters sensitivity to fenretinide of SH-EP1 cells

SH-EP1 cells stably transfected with p75shRNA were found to have approximately 50 % knockdown of p75NTR relative to their scrambled control construct-transfected counterparts (Fig. 3a). SH-EP1 cells were also stably transfected with a p75NTR expression construct to induce overexpression, and the corresponding empty plasmid, respectively. The p75NTR-overexpressing cells expressed approximately 200 % as much p75NTR as their empty plasmid-transfected counterparts (Fig. 3a). As is shown in Fig. 3b, c, twofold knockdown of p75NTR results in decreased sensitivity to fenretinide and decrease in the initiation of apoptotic stimuli. Conversely, twofold overexpression of p75NTR results in a 10–30 % increase in sensitivity to fenretinide.

To ensure that this effect was not unique to the particular shRNA used, we used lentiviral infection with each of six different p75NTR/scrambled shRNAs, each directed at a different segment of the p75NTR mRNA sequence. This resulted in close to 100 % infection as determined by GFP expression (images not shown), and between 60 and 97 % knockdown of p75NTR expression which was quantified using qPCR (Fig. 4a) as well as western blotting (Fig. 4b). Figure 4c shows p75NTR protein expression in N-type neuroblastoma cells (SH-EP1 and SK-N-AS) and S-type neuroblastoma cells (SH-SY5Y). Figure 4d demonstrates that, in the case of the two differentially targeted transductants with the greatest degree of p75NTR knockdown (transductants 5-1 and 6-1), the sensitivity of SH-

EP1 cells to fenretinide (72 h) was decreased as it was in the case of transfection with p75shRNA (Fig. 3).

In order to demonstrate that this effect was not unique to SH-EP1 cells, we studied SK-N-AS (another S-type cell line) and SH-SY5Y (an N-type cell line) cells infected with the lentiviral p75NTR/scrambled shRNA constructs 5-1 and 6-1, found to be most effective in SH-EP1 cells. Once again, knockdown of p75NTR expression decreased the sensitivity of SH-SY5Y (Fig. 4e) and SK-N-AS (Fig. 4f) cells to fenretinide. Note that corresponding with their lower level of native expression of p75NTR (Fig. 4c), empty vector-transfected SH-SY5Y cells are less sensitive to fenretinide than empty vector-transfected SK-N-AS cells, but not completely refractory. This is consistent with the general chemosensitivity of N-type cells relative to S-type cells and points out the importance of p75NTR in determination of chemosensitivity.

Fenretinide-induced apoptosis is accompanied by p75NTR-modulated accumulation of mitochondrial ROS in SH-EP1 cells

Fenretinide has been shown to induce oxidative stress and accumulation of mitochondrial ROS in neuroblastoma cells. We examined the nature of the accumulated species and the effects of p75NTR content on their accumulation in SH-EP1 cells treated with 10 μ M fenretinide.

DCFDA and DHE are intracellular dyes that fluoresce in the presence of OH^- and cytosolic O_2^- radicals, respectively. There was no significant change in OH^- or cytosolic O_2^- concentrations after fenretinide treatment (3–10 μ M; 72 h) in p75NTR knockdown, overexpressing, or control SH-EP1 cells. Similarly, nitric oxide synthase levels did not change in p75NTR knockdown, overexpressing, or control SH-EP1 cells treated with fenretinide (data not shown). In contrast, mitochondrial O_2^- increased within 30 min of fenretinide (shown for 3 μ M; Fig. 5a) exposure in all SH-EP1 cell transfectants and was significantly higher in scrambled shRNA-transfected cells than in p75NTR shRNA-transfected cells. This effect was demonstrable at concentrations between 3 and 10 μ M, as well (data not shown).

In addition, H_2O_2 accumulation was highest in p75NTR-overexpressing cells and lowest in p75NTR knockdown cells, with control transfected cells (i.e., scrambled shRNA and empty plasmid transfectants) exhibiting an intermediate level of H_2O_2 . This effect was robustly reproducible ($n = 4$) but did not reach statistical significance (Fig. 5b).

Scavenging mitochondrial ROS prevents fenretinide-induced apoptosis

DHA and N-acetylcysteine are “prodrugs” of antioxidants. DHA enters the mitochondria via the facilitative glucose transporter 1 and accumulates there as ascorbic acid; N-acetylcysteine is converted to glutathione in the cytoplasm. This enhances cytoplasmic glutathione more and with a shorter latency than mitochondrial glutathione. Pretreatment of p75NTR-overexpressing or control SH-EP1 cells with DHA (400 μ M; 24 h) completely prevented fenretinide-induced (0–20 μ M; 72 h) apoptosis. In contrast, pretreatment with N-acetylcysteine (5 mM; 24 h) did not change the effect of fenretinide on the cells (Fig. 5c).

Inhibition of complex II of the mitochondrial electron transport chain prevents p75NTR-modulated, fenretinide-induced ROS accumulation in and death of SH-EP1 cells

Cuperus et al. [18] suggested that mitochondrial complex II is the major source of fenretinide-induced oxidative stress in neuroblastoma cells. We, therefore, examined the ability of complex-specific inhibitors of mitochondrial electron transport to abolish the

difference in mitochondrial ROS generation and apoptosis induction between p75NTR knockout and control SH-EP1 cells treated with fenretinide.

Figure 5d shows that, while rotenone and antimycin A do not affect p75NTR-modulated, fenretinide-induced cell death (Alamar blue), TTFA converts the response of p75NTR-control SH-EP1 cells to fenretinide to that of p75NTR-knockout cells.

Discussion

p75NTR is one of the several receptors identified as mediating the dependence of cells on neurotrophins for survival. It binds to nerve growth factor, pro-nerve growth factor, brain-derived growth factor, and neurotrophin-3 and functions as both an independent receptor and a co-receptor with tyrosine kinase (Trk) receptors. As an independent receptor, it is variably pro- or anti-apoptotic (reviewed in [21]).

We have previously demonstrated the redox activity of p75NTR in PC12 rat pheochromocytoma cells [9, 10]. p75NTR expression protected PC12 cells from cytoplasmic generation of peroxide, but not from mitochondrial generation of superoxide, after 6-hydroxydopamine treatment; the intracellular domain of p75NTR (p75ICD) is sufficient for this effect. Enhancement of downstream phosphorylation of Akt and phosphoinositol-3-kinase appear to underlie this antioxidant protection [10, 23].

The current studies examine the effects of p75NTR expression on the cytotoxicity of fenretinide, an apoptosis-inducing retinoic acid analogue [16], in neuroblastoma cells. Fenretinide induces accumulation of superoxide in the mitochondria of neuroblastoma cells. Recent studies by others [18] and the data presented herein suggest that it interferes with mitochondrial electron transport to complex II. Expression of p75NTR enhances the cytotoxicity of fenretinide. This enhancement can be completely overcome by interfering with mitochondrial accumulation of ROS, and p75NTR expression alone does not induce apoptosis of neuroblastoma cells, suggesting that p75NTR potentiates mitochondrial susceptibility to fenretinide or fenretinide activity directly, rather than independently and additively inducing apoptosis.

Overexpression of p75NTR enhances fenretinide treatment-associated cytoplasmic accumulation of H_2O_2 as well, but under native conditions, fenretinide-induced generation of H_2O_2 is not robust and administration of N-acetylcysteine, a glutathione precursor, does not diminish fenretinide-induced apoptosis in neuroblastoma cells.

It has been hypothesized that p75ICD functions as a nuclear transcription factor [24]. The effects of fenretinide involve complex II, a mitochondrial complex the four subunits of which are all encoded by nuclear DNA [25], making it plausible that p75ICD affects the synthesis of the proteins that make up complex II.

It is interesting that, within a particular neuroblastoma cell line, downregulation of p75NTR attenuates and upregulation of p75NTR potentiates sensitivity to fenretinide; and across neuroblastoma cell types (i.e., comparing S- and N-type lines), relative p75NTR expression predicts relative sensitivity to fenretinide.

S-type neuroblastoma cells, responsible for residual disease after aggressive treatment of metastatic neuroblastoma [13, 14], and neuroblastoma stem cells ("I-type" cells; [26]) express high levels of p75NTR. Furthermore, their high ratio of p75NTR to TrkA leads to p75NTR signaling that is independent of and separate from TrkA [4]. Fenretinide may therefore be particularly effective in otherwise chemoresistant neuroblastoma. Conversely, overexpression of p75NTR may be a biomarker for fenretinide efficacy specifically in S-

type neuroblastoma cells. Other chemoresistant nervous system tumors express p75NTR in vast excess over Trk family receptors, as well. Medulloblastoma cells overexpress p75NTR, assumed as a marker for the role of persistent embryonal cells in this tumor [6], and those with low TrkC expression are the least responsive to therapy [27]. Furthermore, glioblastoma stem cells overexpress p75NTR, and one study has suggested that p75NTR is a marker for the invasive nature of these cells [28]. In this era of molecular individualization of chemotherapy, the suggestion of differential redox activity of p75NTR in the cytoplasm and the mitochondria, respectively, may guide the choice of chemotherapy by pairing oxidant mechanisms of drugs with p75NTR expression profile in tumors. For example, adjunctive administration of fenretinide with β -phenethyl isothiocyanate, a selective depleter of mitochondrial glutathione [29], might be especially efficacious in tumors that overexpress p75NTR.

The exploitation of mitochondrial redox state in the design of chemotherapeutic strategies has been suggested in other contexts, as well [30]. Drugs that alter mitochondrial stability and function have been termed “mitocans.” Differences between normal and neoplastic mitochondria are thought to underlie an enhanced susceptibility of cancer cells to mitocan-induced perturbations of mitochondrial metabolism and accumulation of ROS. Such differences may permit exploitation of p75NTR-induced potentiation of fenretinide interference with electron transport without induction of systemic toxicity or damage to normal neural tissue.

Finally, small molecule screening techniques might be used to identify compounds that selectively enhance the downstream effectors of p75NTR-induced enhancement of fenretinide efficacy as adjunctive drugs for neural crest tumor therapy.

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Fig. 1.

Fenretinide induces cell death in SH-EP1 human neuroblastoma cells. **a** Concentration–response curve for fenretinide (0–20 μM; 72 h) treatment of SH-EP1 cells. Manual cell counts were performed and the mean $\pm$ SEM of counts from 3 high power fields are shown. Results from a representative experiment of three performed are shown. **b** Metabolic assay for cell viability (*Alamar blue*) demonstrating differential results at 24 (*black squares*) and 72 (*gray circles*) h exposure to fenretinide (0–10 μM). At all concentrations of fenretinide, values at 24 h differ from those for 72 h treatment with $p < 0.01$. **c** Differential lactate dehydrogenase (LDH) release from SH-EP1 neuroblastoma cells treated with fenretinide (0–10 μM) for 24 (*black squares*) and 72 h (*gray circles*). At all concentrations of fenretinide of or above 4 μM, values at 24 h differ from those for 72 h treatment with $p < 0.01$. **d** *Alamar blue* assay showing differential sensitivity to fenretinide of SH-EP1 cells transfected with an expression construct for p75NTR and, therefore, overexpressing the receptor (OE) or a control construct (MOCK). At all concentrations of fenretinide of or above 6 μM, values at 24 h differ from those for 72 h treatment with $p < 0.05$.

Fig. 2.

Fenretinide-induced death of SH-EP1 human neuroblastoma cells is accompanied by concentration- and time-dependent cleavage of poly-ADP ribose polymerase (PARP) and caspase-3 and concentration-dependent nuclear fragmentation and condensation. **a** Concentration-dependent fenretinide-induced cleavage of PARP at 24 h of fenretinide exposure. A representative Western blot is shown. For this and all subsequent graphs, *error bars* signify the SEM of three or more independent values. * $p < 0.05$; ** $p < 0.01$ relative to control; Student's *t*-test. **b** Time course of PARP cleavage in fenretinide-treated (10 μM) SH-EP1 cells. ** $p < 0.01$; *** $p < 0.001$ relative to $t = 0$; Student's *t*-test. **c** Time course of caspase-3 cleavage in fenretinide-treated (10 μM) SH-EP1 cells. * $p < 0.05$ relative to $t = 1$ h; Student's *t*-test. **d** Hoechst dye 33242 staining of SH-EP1 cells following fenretinide treatment (10 μM; 72 h) demonstrates apoptotic nuclear morphology. **e** Concentration dependence of apoptotic nuclear morphology after fenretinide treatment (72 h) in SH-EP1 cells. ** $p < 0.01$; relative to [fenretinide] = 0; Student's *t*-test

Fig. 3.

Knockdown and overexpression of p75NTR in SH-EP1 cells. **a** Western blot demonstrates that shRNA transfection (p75 shRNA) results in 50 % knockdown of p75NTR protein expression relative to scrambled control RNA (Scr), and transfection with a constitutive expression construct for p75NTR (p75OE) results in twofold overexpression relative to transfection with a control empty plasmid (Mock p75OE). The graph depicts mean ± SEM for three independent determinations of optical density of the p75NTR Western blot bands. ****** $p < 0.01$ relative to respective control; Student's t -test. **b, c** Stable knockdown of p75NTR expression with shRNA shifts the fenretinide concentration–response curve of SH-EP1 human neuroblastoma cells. **b** Alamar blue assay showing differential sensitivity to fenretinide of SH-EP1 cells transfected with p75NTR shRNA (white squares) or scrambled control shRNA (gray circles), respectively. At all concentrations of fenretinide, values for p75shRNA-transfected cells differ from those for scrambled RNA-transfected cells with $p < 0.01$. **c** Caspase-3 cleavage is first seen by 24 h of fenretinide exposure in p75shRNA- (black bars) and scrambled shRNA- (white bars) treated cells, but is 50–80 % higher in scrambled transfectants than in p75shRNA knockdown cells. ****** $p < 0.01$ relative to SCR control; Student's t -test

Fig. 4.

p75NTR knockdown by lentiviral transduction in human neuroblastoma cells and sensitivity to fenretinide treatment. **a** Lentiviral transduction of SH-EP1 (S-type) cells with a p75shRNA demonstrates 80–90 % knockdown in the 5-1 and 6-1 transfectants relative to empty vector and scrambled control transductants. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ relative to scrambled RNA-transduced control cells; Student's t -test. **b** p75NTR protein expression in lentiviral knockdown of SH-EP1 cells. Western blot composite showing lentiviral knockdown of p75NTR in SH-EP1 cells with corresponding actin loading controls. **c** p75NTR expression in other neuroblastoma cell lines. Western blot composite showing

p75NTR expression in SH-EP1, SK-N-AS, and SH-SY5Y cells with corresponding actin loading controls. **d** Lentiviral knockdown of p75NTR makes SH-EP1 cells more resistant to fenretinide-induced death than vector and scrambled control transductants. Values for vector-transduced cells are not significantly different from those for scrambled construct-transduced cells. Values for 5-1 cells differ from those for scrambled construct-transduced cells with $p < 0.05$; those for 6-1 cells differ from those for scrambled construct-transduced cells with $p < 0.01$. **e** Lentiviral knockdown of p75NTR in SH-SY5Y (N-type) cells induces resistance to fenretinide treatment relative to empty vector- and scrambled construct-transduced controls. This effect is not apparent below 10 μM fenretinide, assumed because SH-SY5Y and other N-type cells express only very low levels of p75NTR in their native state (see **c**, above). Values for vector-transduced cells are not significantly different from those for scrambled construct-transduced cells. Values for 5-1 and 6-1 cells differ from those for scrambled construct-transduced cells with $p < 0.01$. **f** Lentiviral knockdown of p75NTR in SK-N-AS (S-type) cells also induces resistance to fenretinide treatment relative to empty vector- and scrambled construct-transduced controls. Values for vector-transduced cells are not significantly different from those for scrambled construct-transduced cells. Values for 5-1 cells differ from those for scrambled construct-transduced cells with $p < 0.01$; those for 6-1 cells differ from those for scrambled construct-transduced cells with $p < 0.001$.

Fig. 5.

Induction by fenretinide of accumulation of mitochondrial reactive oxygen species in SH-EP1 human neuroblastoma cells. **a** MitoSOX™ staining of SH-EP1 cells following fenretinide treatment demonstrates accumulation of superoxide in mitochondria within 30 min that persists for at least 3 h of exposure. Superoxide accumulation is more robust in scrambled construct transfectants (*white bars*) than in p75shRNA transfectants (*black bars*). * $p < 0.05$; ** $p < 0.01$ relative to p75shRNA transfectants; Student's t -test. **b** DCFDA staining shows that there is no statistically significant increase in cytosolic H_2O_2 after fenretinide treatment of scrambled construct- or p75shRNA-transfected SH-EP1 cells. There

is, however, a rise in cytosolic H_2O_2 in p75NTR-overexpressing (OE) SH-EP1 cells after fenretinide treatment. **c** Fenretinide-induced cell death is inhibited when SH-EP1 cells are pretreated with dehydroascorbic acid (DHA; $p < 0.01$ relative to medium-treated control) but not N-acetylcysteine (NAC; $p > 0.05$ relative to medium-treated control). **d** Pretreatment of SH-EP1 cells with mitochondrial complex inhibitors before treatment with fenretinide suggests that complex II is a major source of accumulation of mitochondrial reactive oxygen species. Pretreatment with rotenone (complex I inhibitor) and antimycin A (complex III inhibitor) have no effect, but pretreatment with TTFA inhibits ($p = 0.05$ relative to medium-treated control at concentrations of or $>12 \mu M$) the induction of cell death by fenretinide