p38\text{MAPK}-Dependent Sensitivity of Ewing's Sarcoma Family of Tumors to Fenretinide-Induced Cell Death

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Abstract Purpose: There is an urgent need for new therapeutic strategies in Ewing's sarcoma family of tumors (ESFT). In this study, we have evaluated the effect of fenretinide [N-(4-hydroxyphenyl)retinamide] in ESFT models.

Experimental Design: The effect of fenretinide on viable cell number and apoptosis of ESFT cell lines and spheroids and growth of s.c. ESFT in nu/nu mice was investigated. The role of the stress-activated kinases p38\text{MAPK} and c-Jun NH\text{2}-terminal kinase in fenretinide-induced death was investigated by Western blot and inhibitor experiments. Accumulation of reactive oxygen species (ROS) and changes in mitochondrial transmembrane potential were investigated by flow cytometry.

Results: Fenretinide induced cell death in all ESFT cell lines examined in a dose- and time-dependent manner. ESFT cells were more sensitive to fenretinide than the neuroblastoma cell lines examined. Furthermore, fenretinide induced cell death in ESFT spheroids and delayed s.c. ESFT growth in mice. p38\text{MAPK} was activated within 15 minutes of fenretinide treatment and was dependent on ROS accumulation. Inhibition of p38\text{MAPK} activity partially rescued fenretinide-mediated cell death in ESFT but not in SH-SY5Y neuroblastoma cells. c-Jun NH\text{2}-terminal kinase was activated after 4 hours and was dependent on ROS accumulation but not on activation of p38\text{MAPK}. After 8 hours, fenretinide induced mitochondrial depolarization ($\Delta \psi_{\text{m}}$) and release of cytochrome c into the cytoplasm in a ROS- and p38\text{MAPK}-dependent manner.

Conclusions: These data show that the high sensitivity of ESFT cells to fenretinide is dependent in part on the rapid and sustained activation of p38\text{MAPK}. The efficacy of fenretinide in preclinical models demands the evaluation of fenretinide as a potential therapeutic agent in ESFT.

The Ewing's sarcoma family of tumors (ESFT) are small round cell tumors with limited neural differentiation, characterized by the fusion of the 5' portion of the EWS gene with the 3' portion of a gene of the ETS family of transcription factors (1). ESFT constitutes 3% of all pediatric malignancies (2) and are most frequently diagnosed in adolescents and young adults between the ages 10 and 25 years. Treatment intensification has improved prognosis for some patients (2, 3), but 30% to 40% of those with localized and 80% with metastatic disease still die due to disease progression. Recurrence and metastases continue to pose the most difficult challenge for management and treatment of these solid cancers, emphasizing the urgent need for new treatment strategies in ESFT.

Retinoic acid has well-described antiproliferative and differentiation-inducing activities in several malignant cell types and is clinically effective in the treatment of some cancers (e., all-trans retinoic acid in acute promyelocytic leukemia; refs. 4, 5) and 13-cis retinoic acid in disseminating neuroblastoma (6). Unfortunately, the clinical use of retinoic acid in many solid tumors has been restricted by dose-limiting side effects and low therapeutic efficacy; in ESFT, retinoic acid has no effect on cell growth or survival (7, 8). However, the identification of selective retinoic acid derivatives, which are capable of inducing apoptosis and display synergy with other anticancer therapies, promises more effective and less toxic strategies for treatment.

Fenretinide [N-(4-hydroxyphenyl)retinamide] is a synthetic vitamin A analogue with recognized chemopreventive (9) and antitumor activity (10–12). Phase I studies have shown that it is well tolerated in both adults (13, 14) and children (15) and may have efficacy in tumors that do not respond to other retinamides or retinoic acid derivatives. Furthermore, fenretinide acts to additively or synergistically increase the apoptotic response of some cancer cells to chemotherapeutic drugs (16, 17). These important studies suggest that fenretinide may be useful in different clinical situations: increasing response rates to initial therapy by pretreating patients with fenretinide before conventional therapy, targeting disease that develops chemoresistance, treating minimal residual disease, potentially sustaining remission, and preventing recurrence and relapse as a maintenance therapy.
Although the in vivo efficacy of fenretinide has been widely documented, the mechanism by which fenretinide induces cell death is not fully understood. The mode of action of fenretinide and the mode of action of classic retinoids are distinct. Unlike cell death mediated by the classic retinoids, fenretinide-mediated cell death is largely independent of retinoic acid receptors (18, 19), although accumulation of reactive oxygen species (ROS) has been described in many different tumor cell types following exposure to fenretinide (12, 20). Although the mechanisms of fenretinide-mediated ROS production are not well characterized, the mitochondrial electron transport chain (21, 22) and lipoxygenase enzymes (23, 24) have both been implicated. The ability of antioxidants to prevent fenretinide-induced cell death suggests a critical role for ROS in the apoptotic signal transduction (21). Interestingly, ROS activate members of the mitogen-activated protein kinase (MAPK) cascade, including c-Jun NH2-terminal kinase (JNK), p38MAPK, and Big MAPK-1 (25). Furthermore, JNK-dependent, fenretinide-induced cell death has been observed in prostate carcinoma cell lines (26).

Given the efficacy of fenretinide-induced cell death in neuroblastoma and other cancers, the aim of this study was to investigate the effect of fenretinide on ESFT cells in vitro and on s.c. growing human ESFT in nude mice. Because p38MAPK plays a key role in cell death of ESFT cells (27), we have also asked whether this stress-activated kinase mediates the effects of fenretinide on ESFT cells.

**Materials and Methods**

Fenretinide (Janssen-Cilag, Basserdorf, Switzerland) was stored protected from light as a 10 mmol/L stock in ethanol (BDH, United Kingdom) at −20°C. Basic fibroblast growth factor (Sigma, Dorset, United Kingdom) was dissolved in filter-sterilized 1% bovine serum albumin (Sigma) and aliquoted before storing as a stock solution (25 mg/mL) at −20°C. 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-imidazole (SB202190, Calbiochem, San Diego) was protected from light and stored at −20°C as a stock solution of 5 mg/mL in DMSO (Sigma) and used at a final concentration of 20 μmol/L. Vitamin C (L-ascorbic acid, Sigma) was stored at room temperature and used as a 1 mol/L stock in filter-sterilized double-distilled H2O immediately before use. Selective inhibitors of ROS generation pathways were used and stored in ethanol and were purchased from Calbiochem unless otherwise stated. Ketoconazole, a cytochrome P450 inhibitor, was used at a final concentration of 1 μmol/L. The mitochondrial respiratory chain inhibitor AEBSF (Sigma) was used as a stock solution in DMSO and used at a final concentration of 10 μmol/L. The NADPH oxidase inhibitor diaphenyleneiodonium was stored in DMSO and used at a final concentration of 10 μmol/L. The NADPH oxidase inhibitor diphenyleneiodonium was stored in DMSO and used at a final concentration of 10 μmol/L. Nordihydroguaiaretic acid, a pan-lipoxygenase inhibitor, was used as a stock solution of 50 μmol/L. The selective 5-lipoxygenase inhibitor MK886 was used at a final concentration of 1 μmol/L. Caffeic acid, a selective 5- and 15-lipoxygenase inhibitor, was used at a final concentration of 10 μmol/L. 15-Lipoxygenase was selectively inhibited using PD146176 at 0.3 μmol/L and was a gift from Parke-Davis Pharmaceutical Research, Ann Arbor, MI. The 12-lipoxygenase-selective inhibitor baicalin was used as a final concentration of 1 μmol/L. N-Vanillyl-nonanamide, a NADPH oxidase inhibitor, was used at 10 μmol/L (vehicle methanol) and was purchased from Sigma. The mitochondrial respiratory chain inhibitor AEBBF (Sigma) was used at final concentration of 100 μmol/L (in H2O). The ROS-responsive dye CM-DCFDA (Molecular Probes, Eugene, OR) was prepared in DMSO as a 5 μmol/L stock solution, placed on ice, and used immediately. H2O2 (Sigma) was prepared as a 100 mmol/L stock solution in double-distilled H2O and used immediately. 3′,3′,5′-Dihexylxocarboxyacine (Sigma) was stored as a stock solution in DMSO (5 mmol/L) and used at a final concentration of 40 mmol/L. Anti-human polyclonal (ADP-ribose) polymerase (PARP) antibody (BD PharMingen/Becton Dickinson, Oxford, United Kingdom) was used at a final concentration of 1:500. Anti-human tubulin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at 1:5,000. Anti-human total p38 (rabbit polyclonal), phospho-p38, total JNK (rabbit polyclonal), phospho-JNK, and cytochrome c (rabbit polyclonal) antibodies were purchased from Cell Signaling (Hertfordshire, United Kingdom) and used at 1:1,000. All antibodies were mouse monoclonal unless otherwise stated. Alexa Fluor 680 IgG rabbit anti-mouse and goat anti-rabbit secondary antibodies (Molecular Probes) were used at 1:5,000. Antibodies purchased from Cell Signaling were stored at −20°C; all other antibodies were stored at 4°C.

**Cell culture.** The substrate adherent ESFT cell lines TC-32, RD-ES, and TTC-466 were grown in RPMI 1640 (Sigma) containing 10% FCS (SeraLab, Sussex, United Kingdom); medium for TTC-466 cells was supplemented with 10% tumor cell conditioned medium. A673 cells were grown in DMEM (Sigma) and 10% FCS, SK-ES1 cells in McCoy's medium (Sigma) plus 15% FCS, and SK-N-MC and SH-SY5Y cells in DMEM/F-12 plus 10% FCS. SH-EP1 and SK-N-SH cells were grown in a 1:1 mix of DMEM and MEM and 10% FCS. All cell lines were purchased from the American Type Culture Collection, Manassas, VA, except for the following cells that were kind gifts: TC-32 cells from Dr. J. Toretsky (Division of Pediatrics, University of Maryland, Baltimore, MD), the TTC-466 cells from Dr. P. Sorensen (British Columbia Children's Hospital, Vancouver, British Columbia, Canada), and the SH-SY5Y cells from Dr. R.A. Ross (Fordham University, Bronx, NY). Spheroid cultures were initiated by seeding cells into flasks coated with 1.4% agar (Sigma); after 3 to 4 days, cell aggregates were transferred to Integra Bioscience, Switzerland spinner flasks (100 or 250 mL); spheroid formation was promoted and preserved by incubation in spinner flasks with continuous stirring (15 × g). Medium was replenished every 3 days by allowing the spheroids to settle in the bottom of the flask, aspirating 80% of the used medium and replacing with fresh medium. All incubations and treatments of cells, unless otherwise stated, were carried out in a humidified atmosphere of 5% CO2, 95% air at 37°C (Sanyo Gallenkamp, Loughborough, United Kingdom).

**Viable cell counts.** Cells were seeded into Primaria six-well plates (Fahrenheit, Leeds, United Kingdom), maintained for 24 hours, and treated with fenretinide (0-10 μmol/L) for 0 to 24 hours. Control cultures were treated with ethanol vehicle. Substrate adherent and nonadherent cells were harvested by trypsinization and centrifugation and resuspended in 1 mL normal growth medium, and viable cell number was counted using the Vi-cell automated trypsin blue exclusion assay (Becton Dickinson). To assess spheroid viability and growth characteristics after treatment with fenretinide (3-10 μmol/L, 24-48 hours), spheroids were incubated in 100 mL medium, spheroid slurry was allowed to settle for 5 minutes, and spheroid-free medium (90 mL) was removed and discarded. Three aliquots (2 mL) of spheroid slurry were collected while gently agitating the spheroid suspension to ensure even sampling. Spheroids were either fixed in 4% formal saline for 30 minutes, suspended in 1.4% agar and paraffin embedded for the preparation of sections for staining (see Immunohistochemistry), or disaggregated using EDTA and trypsin to generate a single cell suspension that was analyzed using the Vi-cell automated trypsin blue exclusion assay for total and viable cell number.

**In vivo methodology.** The effect of fenretinide (100 mg/kg/d s.c. or 5 days treatment, 2 days rest by oral gavage) on s.c. ESFT growth was examined in nu/nu mice. Mice (n = 18) were injected in one (n = 10) or both (n = 8) flanks s.c. with a single cell suspension of RD-ES cells (2.5 × 107 in 0.2 mL medium). On day 15, after the development of a palpable tumor, mice were (a) injected with either vehicle alone or fenretinide or (b) treated by oral gavage with fenretinide or vehicle. A stock solution of fenretinide (250 mg/mL) was prepared in ethanol and diluted in 0.9% saline for s.c. injection into mice or in corn oil and...
cremaphor for oral gavage (28). Tumor size was measured twice weekly and mice were sacrificed when s.c. tumors reached a size of 1.4 cm², at the end of the experiment, or if the mouse showed signs of distress.

Administration of fenretidine to the test group was stopped when all mice in the control group had been sacrificed. Tumors were excised, fixed in 10% phosphate-buffered formalin, and paraffin embedded for the preparation of tissue sections.

**Immunohistochemistry.** Sections (3 μm) of paraffin-embedded spheroids or RD-ES tumors were heat sealed (1 hour) onto glass slides. Sections were deparaffinized with H&E and morphology was observed by light microscopy (Zeiss Axiosplan microscope, United Kingdom). Apoptosis was detected using the ApopTag Apoptosis Detection kit (Chemicon, Temecula, CA) according to the manufacturer’s instructions. Proliferation was detected using the Apoptag Apoptosis Detection kit (Chemicon, Temecula, CA) according to the manufacturer’s instructions. Proliferation was detected using the Apoptag Apoptosis Detection kit (Chemicon, Temecula, CA) according to the manufacturer’s instructions.

**Inhibition of p38MAPK using SB202190.** The cell-permeable p38MAPKα and p38MAPKβ selective inhibitor SB202190 was used to assess the role of p38MAPK in fenretidine (1.5 μmol/L)–induced cell death. Cells were incubated with SB202190 (20 μmol/L) for 1 hour at 37°C before addition of fenretidine. Control cells were treated with vehicle DMSO.

**Cellular fractionation.** After incubation with fenretidine (1.5 mol/L, 0-24 hours), cells were placed on ice and washed once with ice-cold PBS (2 mL), fractionation buffer was added (2 mL), and the cell monolayer was disrupted using a cell scapper. The cell suspension was centrifuged at 11,600 x g for 5 minutes (room temperature), and the cell pellet was resuspended in fractionation buffer [0.3 mol/L sucrose, 10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA]. Cells were resuspended and incubated on ice for 5 minutes before passing through a 25 gauge needle. Samples were then centrifuged at 1,000 x g for 10 minutes at 4°C. The supernatant was removed and stored on ice for further fractionation. The cell pellet was washed in fractionation buffer and centrifuged [1,000 x g, 10 minutes, 4°C] to form a cell pellet, which contained the nuclear protein. The supernatant was centrifuged [100,000 x g, 1 hour, 4°C], and the resulting low-density supernatant containing the cytoplasmic proteins was removed. The high-speed centrifugation cell pellet was washed in fractionation buffer (100,000 x g, 1 hour, 4°C) and the resulting supernatant was aspirated and discarded to leave a pellet containing the mitochondrial and plasma membranes; this was resuspended in fractionation buffer. An equal volume of 2 × SDS nonreducing loading buffer [100 mmol/L Tris-HCl (pH 6.8), 20% (w/v) glycerol, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue] at −20°C. Before storage, samples were heated to 99.9°C for 5 minutes (Hybond, Omegine, Cambridge, MA) and placed quickly on ice. Protein concentration was determined using the Bio-Rad DC Protein Assay kit (Bio-Rad, Herts, United Kingdom). To detect protein phosphorylation and PARP cleavage, Western blotting was carried out using the Li-cor Odyssey IR imaging system (Lincoln, NE).

**Detection of mitochondrial membrane permeability transition.** Induction of mitochondrial membrane permeability transition was evaluated by measuring ΔΨm. After treatment with fenretidine (0-10 μmol/L), both floating and attached cells were collected and pelleted (1,000 x g, 5 minutes, room temperature) followed by incubation with 3,3’-dihexyloxacyarbonyl (40 nmol/L, 15 minutes, 37°C in PBS). The uncoupler of the mitochondrial respiratory chain carbonyl (CCCP) (50 μmol/L, 1 hour) was used as a positive control for transmembrane potential loss. Fluorescence was analyzed using flow cytometry (FACS caliber, excitation 488 nm, emission 552 nm): 10,000 events were analyzed per sample.

**Statistical methods.** The logarithm of the percentage of viable cell number was compared between SH-SY5Y and the six ESFT cell lines for increasing concentrations of fenretidine; ANOVA was used to fit linear regression trends to the logarithm of the percentage of viable cells in control cultures against the logarithm of fenretidine concentration, looking at different slopes for different cell lines. The SH-SY5Y cell line was used for this comparison, as this was the most sensitive of the three neuroblastoma cell lines studied. Tests for nonlinear effects were performed. The effect of fenretidine on spheroid cultures was assessed using a factorial ANOVA on the logarithm of the viable cell number, with factors for fenretidine concentration, cell line, and time included. The interactive effects of fenretidine in the s.c. tumor growth mouse model were assessed by comparing the logarithm of tumor size between the treatment and control groups by a repeated-measures
analysis, fitting a random linear trend over time and intercept for each mouse and then superimposing an overall linear trend over time, group intercept, and group trend. The group effects were compared and departures from group trends were examined for individual mice.

The effect of fenretinide alone and in combination with the inhibitor SB202190 was assessed by an ANOVA on the logarithm of the viable cell number, with factors for presence of inhibitor SB202190 and concentration of fenretinide included. t tests were performed to evaluate whether viable cell number was different between control and fenretinide at a concentration of 0.7 to 10 μmol/L with or without SB202190. An ANOVA of the factors fenretinide concentration and SB202190 was carried out, fitting a linear regression effect to log(fenretinide concentration) but also including terms for further nonlinear effects in fenretinide and SB202190. Tests were carried out to determine whether there was an overall decrease in viability with fenretinide, whether this decrease was similar in the presence or absence of SB202190 and whether the decrease was linear in log(fenretinide concentration), or whether there were significant nonlinear effects.
The logarithm of ROS was analyzed by a factorial ANOVA, with factors for fenretinide (no/yes) and vitamin C (no/yes). The same analysis was carried out on the logarithm of viable cell number, again testing the main effects of fenretinide and vitamin C as well as their interaction. For each of the four combinations of the factors fenretinide and vitamin C, an estimate was produced of the ratio of ROS production to viable cell number. The effect of specific inhibitors of ROS on viable cell number after exposure to fenretinide was analyzed by ANOVA (mixed model to allow for imbalance in treatment factorial combinations). The primary interest of these experiments was the rescue of viable cell number in cell lines treated with inhibitor plus fenretinide compared with cells treated with fenretinide alone. Bias-corrected estimates of differences in rescue among inhibitors were produced by considering the inhibitors as random effects in a mixed model.

Results

**Induction of cell death in Ewing's sarcoma family of tumors by fenretinide in vitro and in vivo.** Fenretinide decreased viable cell number in all six ESFT cell lines studied within 24 hours (IC_{50} range, 0.60-1.25 μmol/L; mean, 0.86 μmol/L; P < 0.001; Fig. 1A). The decrease in viable cell number after treatment with fenretinide reflected an increase in apoptosis, shown by labeling of cells with Annexin V-PI (Fig. 1B). The increase in apoptotic cell fraction was time dependent; the apoptotic fraction in TC-32 cells was 15%, 16%, 38%, and 58% after 0, 8, 16, and 24 hours of treatment, respectively. This was accompanied by cleavage of PARP in TC-32 cells after 8-hour treatment with fenretinide (Fig. 1D), an early event in apoptosis that is caspase-3 dependent. Fenretinide also induces apoptosis in neuroblastoma cells, although the sensitivity of these cells varies considerably (17, 20). All the ESFT cell lines were more sensitive to fenretinide-induced cell death than the neuroblastoma cell lines studied (IC_{50}, 2.25 μmol/L for SH-SY5Y, 8.15 μmol/L for SK-N-SH, and IC_{50} not reached for SH-EP1; Fig. 1A). Collectively, these data show that fenretinide is a potent inducer of apoptosis in ESFT cell lines and that in vitro the ESFT cells are more sensitive to fenretinide than the three neuroblastoma cell lines examined. The mechanism of fenretinide-induced apoptosis has been extensively examined in the neuroblastoma SH-SY5Y cells (23, 24, 30); therefore, we have used these cells to compare the mechanism of response with ESFT cells.

All the cell lines examined produced three-dimensional spheroid cultures that increased with time. Histologic examination of H&E-stained sections of all cell line spheroids showed a small round cell morphology; in RD-ES and SK-ES-1, spheroid rosettes are visible, consistent with ESFT histology. All spheroids form an organized structure of outer proliferative rim and inner necrotic core. Treatment of ESFT spheroids with fenretinide (3-10 μmol/L) resulted in a dose-dependent increase in cell death at 48 hours (P = 0.002); after 72 hours, there were no viable cells in the ESFT spheroid cultures (Fig. 1E). Induction of death was delayed (48 hours) in spheroids compared with that in substrate adherent cultures (24 hours). Fenretinide also induced cell death in the SH-SY5Y spheroids, although this was only significant in spheroids treated for 72 hours (P = 0.002; Fig. 1E). An increase in apoptotic cell number, detected using terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL), was observed at 24 and 48 hours after treatment (Fig. 1F); cell proliferation, detected by staining for Ki-67, was unchanged (data not shown).

Subcutaneous injection of nu/nu mice with a RD-ES cell suspension resulted in rapid tumor growth; after 37 days, 100% of the mice in the control group had tumors of 1.4 cm 2.
were sacrificed according to experimental protocol. The rate of tumor growth was reduced in mice treated with fenretinide (100 mg/kg/d) compared with that in control mice treated with vehicle alone (P = 0.04 for s.c. treated group; Fig. 2A and B). However, there was considerable intermouse variation in the slope and intercept variable estimates, particularly in mice treated with fenretinide s.c.; mouse 6 (s.c. fenretinide treatment group) had a low intercept but faster growth than average for the whole test group. Tumors from mice treated with fenretinide (with the exception of mouse 6 from the s.c. group) showed evidence of apoptosis and necrosis when histology was examined by light microscopy; this is in contrast to all tumors from both control groups (Fig. 2C). Previous studies have suggested that fenretinide may inhibit tumor growth in part through inhibition of angiogenesis (31); this requires investigation in ESFT.

**Fenretinide increased accumulation of reactive oxygen species in Ewing’s sarcoma family of tumor cells.** Fenretinide-mediated cell death is reported to be dependent on ROS accumulation in several different cancer cell lines, including neuroblastoma, leukemia, and prostate cancer (21, 30–33). In TTC-466, SK-N-MC, and TC-32 cells, levels of ROS rapidly increased in a dose-dependent manner after exposure to fenretinide (Fig. 3A). Accumulation of ROS was rapid, and an 8-fold increase was observed after just 5 minutes of fenretinide treatment in TC-32 cells. Exposure to fenretinide (0.7-10 mol/L) induced ROS accumulation that was 2.4-fold higher in TC-32 cells compared with that in the SH-SY5Y cell line (P < 0.001; Fig. 3A); no accumulation of ROS was detected in SH-EP1 cells. The magnitude of ROS accumulation in ESFT relative to neuroblastoma cells may in part account for the increased sensitivity of these cell lines to fenretinide in vitro.

To determine whether ROS production is an effector of fenretinide-mediated death in ESFT cells, we pretreated TC-32 cells with the antioxidant vitamin C (100 μmol/L, 1 hour). This prevented fenretinide-induced (1.5 μmol/L, 1 hour) ROS accumulation (P < 0.001; Fig. 3B). Furthermore, vitamin C inhibited 100% of fenretinide-mediated cell death (1.5 μmol/L, 24 hours) in TC-32 cells (P < 0.001; Fig. 3C). These results suggest that ROS generation is a critical component of the fenretinide-induced death cascade in TC-32 cells. However, vitamin C is a pan-ROS inhibitor and therefore does not identify the mechanism of ROS accumulation in the cell.

The effect of a range of enzyme inhibitors on fenretinide-induced death was evaluated in an attempt to identify a common cellular pathway of fenretinide-mediated ROS generation in ESFT cells. However, the three-factor interaction of inhibitor, cell line, and treatment was highly statistically significant (P < 0.0001), demonstrating that the effects of the inhibitors were dependent on the cell line studied. Strikingly, inhibition of 12-lipoxygenase by baicalein failed to abolish ROS production and did not rescue all the ESFT cell lines from fenretinide-induced cell death, although it did rescue a proportion of TC-32 and SK-ES1 cells. This observation shows that 12-lipoxygenase is not the only mechanism of fenretinide-induced ROS accumulation in ESFT cells, in contrast to its important role in the neuroblastoma cell line SH-SY5Y (23, 24). Interestingly, inhibitors of 5- and 15-lipoxygenase activity also rescued some ESFT cells from fenretinide-induced cell death. In SK-N-MC, SK-ES1, and A673 cells, a proportion of cells were rescued from cell death using inhibitors targeting more than one pathway of ROS generation.

**Fenretinide induces sustained phosphorylation of the stress-activated kinase p38MAPK.** We have shown recently that p38MAPK is a rate-limiting kinase in ESFT cell death (27), and because ROS accumulation primarily initiates stress responses, we have examined the role of this stress-activated kinase in fenretinide-induced cell death. Fenretinide induced phosphorylation of p38MAPK in TC-32 within 15 minutes of exposure to fenretinide (1.5 μmol/L; Fig. 4A). In contrast, activation of p38MAPK first occurred in SH-SY5Y cells 4 hours after

![Fig. 2](https://www.aacrjournals.org/doi/10.1182/clinres.2005.11.8.3141)**Effect of fenretinide on s.c. growth of ESFT in nude mice.** A, mice (n = 10) were injected s.c. in one flank with RD-ES cells (2.5 × 10^6 per mouse). From day 15, when tumor mass was measurable, the mice were treated by s.c. injection daily with either fenretinide (100 mg/kg) or vehicle control. Treatment continued until the end of the experiment (day 37). Points, average tumor size; bars, SEM. The rate of tumor growth was significantly slower in the group of mice treated s.c. with fenretinide compared with vehicle-only controls (P = 0.04); one mouse (F5) showed no decrease in tumor growth. B, mice (n = 8) were injected s.c. in both flanks with RD-ES cells (2.5 × 10^6 per mouse). From day 15, mice were treated by oral gavage with fenretinide (100 mg/kg) in corn oil and cremophor (28). Mice were treated on a cycle of 5 consecutive days on, 2 days off until the end of the experiment. Points, average tumor size; bars, SEM. The rate of tumor growth was slower in the group of mice treated orally with fenretinide compared with vehicle-only controls. In all *in vivo* experiments, mice were weighed and tumor size was measured twice weekly.
incubation with fenretinide (Fig. 4B), although this was sustained to 8 hours following treatment. The activation of p38MAPK in TC-32 cells was enhanced and sustained to 8 hours following treatment. Phosphorylation of p38MAPK was dose dependent, and levels of phosphorylated protein were increased after treatment of TC-32 cells with 1.5 to 10 μmol/L fenretinide for 15 minutes (Fig. 4C). The sustained activation of p38MAPK was also observed in SK-N-MC and TTC-466 cell lines and occurred within 30 and 15 minutes, respectively (data not shown). Pretreatment of TC-32 cells with vitamin C (100 μmol/L, 1 hour) inhibited the fenretinide-induced phosphorylation of p38MAPK (Fig. 4D). Because vitamin C rescued TC-32 cells from fenretinide-induced cell death (Fig. 3C), these observations suggest that p38MAPK may play a role in fenretinide-induced death of ESFT cells and that p38MAPK activation is a consequence of ROS accumulation.

To investigate the role of p38MAPK, we have used the pyridinyl imidazole inhibitor SB202190, an inhibitor of both p38α and p38β kinase activity; this compound has no effect on the activity of JNK or extracellular signal-regulated kinase under the conditions used.3 SB202190 inhibits the activity of p38MAPK by competing for the ATP binding sites of the α and β isoforms (32).

Decreased phosphorylation of p38MAPK after pretreatment of TC-32 cells with SB202190 correlated with a decrease in kinase activity (shown by reduced phosphorylation of activating transcription factor-2; data not shown). Inhibition of p38MAPK activity by pretreatment of cells with SB202190 (20 μmol/L, 1 hour) significantly reduced (P < 0.001) fenretinide-mediated cell death in TC-32, SK-ES1, and RD-ES cells at 1.5 and 3 μmol/L (Fig. 5A) and in TC-32 and SK-ES1 cell lines at 10 μmol/L. Furthermore, inhibition of p38MAPK abolished PARP cleavage in TC-32 cells (Fig. 5C). However, pretreatment of SH-SY5Y cells with SB202190 had no effect on fenretinide-induced cell death (Fig. 5B) and did not prevent cleavage of PARP (Fig. 5C). These data suggest that p38MAPK activation plays an important role in fenretinide-induced cell death of ESFT cell lines but not of SH-SY5Y cells and is consistent with the hypothesis that the mechanism of cell death in ESFT is distinct to that in SH-SY5Y neuroblastoma cells.

Delayed phosphorylation of c-Jun NH2-terminal kinase after exposure to fenretinide. We have shown that fenretinide induces phosphorylation of p38MAPK and that p38MAPK contributes to cell death in ESFT cells. However, because inhibition of p38MAPK did not fully rescue fenretinide-mediated cell death, we hypothesized that the stress-activated kinase JNK may also contribute to fenretinide-mediated apoptosis. Activation of JNK was assessed using Western blot for phosphorylated protein. In contrast to p38MAPK, JNK was not activated 15 minutes after exposure to fenretinide (0.35-10 μmol/L; Fig. 4C), consistent with the hypothesis that p38 MAPK but not JNK is an initiator of fenretinide-induced cell death in ESFT cells. JNK activation was observed 4 hours after the addition of fenretinide (1.5 μmol/L; Fig. 4B) in TC-32 but not in SK-N-MC cells (data not shown), suggesting that JNK activation may play an important effector role, possibly amplifying the cell death cascade in a cell line–dependent manner. As predicted, the vehicle control, SB202190, and vitamin C alone had no effect on the phosphorylation of JNK. Inhibition of p38MAPK by pretreatment of cells with SB202190 before exposure to fenretinide had no effect on JNK activation, suggesting that JNK activation is independent of p38MAPK (Fig. 4E). Not surprisingly, inhibition of ROS accumulation using vitamin C prevented JNK activation and suggests that ROS directly or indirectly regulates JNK activation in TC-32 cells.

Effect of fenretinide on cytochrome c release and $\Delta \psi_m$.

Fenretinide has been reported to induce cytochrome $c$ release and mitochondrial permeability transition in various cancer cell lines, including ESFT cells (33). Therefore, we have determined $\Delta \psi_m$ and cytochrome $c$ release in response to fenretinide in ESFT cells. Treatment of TC-32 cells with fenretinide (1.5 $\mu$mol/L) for 8 hours resulted in release of cytochrome $c$ into the cytoplasm, which increased following 16- and 24-hour treatment, consistent with permeability transition and accumulation of cytoplasmic cytochrome $c$ (Fig. 6A). Cytochrome $c$ release is often, although not exclusively, accompanied by $\Delta \psi_m$ and permeability transition. To assess $\Delta \psi_m$ after fenretinide treatment, the fluorescent dye 3,3'-dihexyloxocarbocyanine was used. TC-32 cells showed a time- and dose-dependent loss of $\Delta \psi_m$ (Fig. 6B and C). However, increases in $\Delta \psi_m$ were low and a $<2$-fold increase in $\Delta \psi_m$ was observed after 8-hour treatment with 1.5 $\mu$mol/L fenretinide. Pretreatment of TC-32 cells with either vitamin C or SB202190 resulted in complete inhibition of $\Delta \psi_m$ (Fig. 6D). These data suggest that the accumulation of ROS and phosphorylation of p38$^{\text{MAPK}}$ precedes the induction of permeability transition. As p38$^{\text{MAPK}}$ is downstream of ROS production, it is likely that signaling from this kinase results directly or indirectly in cytochrome $c$ release and $\Delta \psi_m$, leading to the induction of apoptosis.

Discussion

In this study, we show that fenretinide induced cell death in all substrate adherent ESFT cell lines studied and was characterized by the induction of ROS, PARP cleavage, cytochrome $c$ release, and $\Delta \psi_m$. ESFT cells were up to 4-fold more sensitive (assessed using IC$_{50}$) to fenretinide-induced death than the neuroblastoma cell line SH-SY5Y in vitro. The induction of cell death in ESFT was dependent on an early activation of the stress-activated kinase p38$^{\text{MAPK}}$ and may be subsequently amplified through activation of JNK. This is in contrast to the mechanism of apoptosis induced in SH-SY5Y neuroblastoma cells. Perhaps most importantly for its clinical potential in the treatment of ESFT, fenretinide delayed the growth of ESFT cells in a nude mouse model.
The high sensitivity of ESFT cells to fenretinide suggests this retinoid may be clinically effective against ESFT. The steady-state plasma level of fenretinide achieved in children after administration of the maximum tolerated dose is reported to be \( \sim 10 \) \( \mu \)mol/L (15). Although this may be insufficient to achieve maximum cell kill in some cancer types, it is in excess of the doses that induce death of ESFT cells in vitro. The clinical potential of fenretinide for ESFT is supported by the nude mouse experiments in which there was a marked reduction in growth rate of s.c. RD-ES tumor. No response was observed in one mouse following s.c. injection with fenretinide, but this may reflect limitations in the bioavailability of the fenretinide and delivery route used. Improved response may be further achieved by the delivery of fenretinide in combination with inhibitors of ceramide metabolism (33, 34), with conventional chemotherapeutic agents (35), or with agents that inhibit cellular antioxidant activity (see below). Intravenous and improved oral formulations of fenretinide might further improve bioavailability and clinical response (33).

Exposure of ESFT cell lines to fenretinide resulted in a rapid accumulation of ROS to levels greater than those in neuroblastoma cells under similar conditions; this may explain the greater

Fig. 4. Phosphorylation of p38 \( \text{MAPK} \) after treatment of substrate adherent ESFT cells with fenretinide. A, phosphorylation of p38 \( \text{MAPK} \) in TC-32 cells treated with fenretinide (1.5 \( \mu \)mol/L) for 0 to 120 minutes examined by Western blot. Untreated cells were incubated with vehicle for 120 minutes. B, phosphorylation of p38 \( \text{MAPK} \) and JNK in TC-32 and SH-SYSY cells treated for 0 to 8 hours and examined by Western blot. Untreated cells were incubated with vehicle for 8 hours. C, phosphorylation of p38 \( \text{MAPK} \) and JNK after 15-minute treatment in TC-32 cells with fenretinide (0-10 \( \mu \)mol/L). D, effect of vitamin C – dependent inhibition of ROS on p38 \( \text{MAPK} \) phosphorylation in TC-32 cells after treatment with fenretinide. Cells were either untreated, treated with fenretinide alone (1.5 \( \mu \)mol/L), pretreated with vitamin C (100 \( \mu \)mol/L) for 1 hour and then treated with fenretinide (1.5 \( \mu \)mol/L) alone. After treatment for 6 hours, cells were harvested and protein extracts were prepared, separated by SDS-PAGE, and analyzed by Western blot for phosphorylated and total p38 \( \text{MAPK} \) proteins. E, JNK phosphorylation in TC-32 cells were either untreated, treated with vitamin C (100 \( \mu \)mol/L, 1 hour), pretreated with vitamin C (100 \( \mu \)mol/L, 1 hour) and then treated with fenretinide (1.5 \( \mu \)mol/L), treated with SB202190 (20 \( \mu \)mol/L, 1 hour) or pretreated with SB202190 (20 \( \mu \)mol/L, 1 hour) and treated with fenretinide (1.5 \( \mu \)mol/L). After 24 hours, cells were harvested, proteins were extracted and size separated by SDS-PAGE, and blots were probed for phosphorylated and total JNK protein. Basic fibroblast growth factor-treated TC-32 cells (20 ng/mL, 15 minutes) were used as a positive control. PO\(^4\)/C0 p38 \( \text{MAPK} \), phosphorylated p38 \( \text{MAPK} \) protein; Total p38 \( \text{MAPK} \), total p38 \( \text{MAPK} \) protein; PO\(^4\)/C0 p54 JNK and PO\(^4\)/C0 p46 JNK, phosphorylated JNK protein; p54 JNK and p46 JNK, total JNK protein.
sensitivity of ESFT cells to fenretinide. The importance of an oxidative pathway is shown by a reduction in ROS accumulation and the rescue of ESFT cells from fenretinide-induced cell death after incubation with the antioxidant vitamin C. However, the mechanism of ROS accumulation in ESFT cells is different from that in SH-SY5Y cells; selective inhibitors of ROS-generating enzymes show that the induction of ROS in ESFT cells was not necessarily dependent on 12-lipoxygenase as described in SH-SY5Y cells (23, 24). The inability of one selective inhibitor to prevent cell death and ROS production in all ESFT cell lines suggests cell line heterogeneity and multiple routes of ROS generation in ESFT after exposure to fenretinide. The initiating event leading to ROS generation following exposure to fenretinide in ESFT remains unknown. However, accumulation of ROS, and therefore responsiveness to fenretinide, will also depend on the constitutive expression and activity of cellular redox enzymes, such as thioredoxin and glutathione S-transferases (36). These detoxification enzymes usually prevent ROS-induced death by scavenging ROS; however, if these defense mechanisms are inadequate, oxidative stress and, in extreme cases, cell death may occur. Consistent with this hypothesis, fenretinide-induced generation of ceramide has been shown to increase oxidative activity by inhibition of the ROS scavenging ability of catalase (37). These observations suggest agents that selectively inhibit cellular antioxidants, which maintain the redox environment in a highly reduced state, may increase the level of ROS accumulation, and enhance fenretinide-induced cell kill.

Fig. 5. Effect of the p38 MAPK inhibitor SB202190 on fenretinide-induced cell death. A, TC-32, SK-ES1, and RD-ES cells were pretreated with SB202190 (+; 20 μmol/L) or DMSO (-) vehicle control for 1 hour before addition of fenretinide (0-10 μmol/L). Viable cell number was determined after 24 hours using the Vi-cell automated trypan blue exclusion assay (n = 6); bars, 95% CI. TC-32 and SH-SY5Y cells were pretreated with SB202190 (20 μmol/L) for 1 hour or DMSO vehicle control before addition of fenretinide (1.5 μmol/L). After 24 hours, cells were harvested and apoptosis assayed after staining with Annexin V and PI by flow cytometry; 10,000 events were counted for each analysis. Cells staining for Annexin V only are early apoptotic (bottom right quadrant), cells staining with Annexin V and PI are late apoptotic (top right quadrant), and those staining with PI alone are end-stage apoptotic or necrotic (top left quadrant). Representative of six independent observations. Percentage of dead or dying cells (sum of Annexin V, Annexin V and PI, and PI-positive cells). B. PARP cleavage was detected by Western blot. TC-32 and SH-SY5Y cells were either untreated, treated with fenretinide alone (1.5 μmol/L), treated with SB202190 (20 μmol/L) for 1 hour and then treated with fenretinide (1.5 μmol/L), or treated with SB202190 (20 μmol/L) alone. After 24 hours, cells were harvested, proteins were extracted and size separated by SDS-PAGE, and blots were probed for PARP. Arrow, 85-kDa PARP cleavage product. Equal protein on the blot was confirmed by hybridization for β-tubulin.
Cellular response to ROS is dependent in part on signal intensity; moderate oxidative stress induces apoptosis and concomitant activation of caspases, whereas necrosis and suppression of the caspases occurs when cells are exposed to higher levels of ROS (38). The major source of ROS in eukaryotic cells in vivo is the mitochondrial respiratory chain components (25), which are essential for controlling the decision of the cell to enter an apoptotic or necrotic process. In ESFT cells, fenretinide induced a dose- and time-dependent increase in \( \Delta \psi_{m} \), suggesting that fenretinide may initiate changes in permeability transition, consistent with the release of cytochrome \( c \) from the intermemochondrial space into the cytoplasm. However, accumulation of ROS occurred earlier (within 5 minutes) than changes in the mitochondrial transmembrane potential (2–8 hours), suggesting that these changes in potential are due to the direct action of fenretinide-induced ROS to open mitochondrial transition pores and release proapoptotic factors and not responsible for the initial rapid accumulation of ROS. Nevertheless, it is clear that changes in the mitochondria are a downstream consequence of fenretinide treatment of ESFT cells and may result in the amplification of the death cascade. This cascade may be amplified by accumulation of further ROS produced from the ubiquinone pool within mitochondria, an important target for ceramide (39–41), or accumulation of specific lipids or glycolipids (42) at the mitochondrial membranes to induce permeability transition and release of proapoptotic factors. Proapoptotic and antiapoptotic members of the Bcl-2 family control mitochondrial transition pores in many systems (43, 44), although Bcl-2, Bax, and Bcl-x\(_{L} \) do not seem to participate in fenretinide-induced death (22, 45). However, this remains controversial (23, 46) and we cannot currently exclude this possibility in ESFT.

ROS can directly or indirectly modulate the functions of many enzymes and transcription factors, which ultimately result in gene expression changes that influence cells to survive or die (45, 47). These include the stress-activated kinases p38MAPK and JNK (46, 47). Inhibition of the rapid and
sustained phosphorylation of p38MAPK after pretreatment of ESFT cells with the antioxidant vitamin C suggests that fenretinide-induced phosphorylation of p38MAPK is downstream of ROS generation. Furthermore, inhibition of p38MAPKα and p38MAPKβ using the cell-permeable and selective inhibitor SB202190 rescued cells from fenretinide-mediated cell death, consistent with the hypothesis that activation of p38MAPK is an important effector of death in ESFT cells. Importantly, activation of p38MAPK in ESFT cells after treatment with fenretinide was rapid and sustained, in contrast to its late activation in SH-SY5Y cells. Pretreatment with SB202190 had no effect on the accumulation of ROS after fenretinide treatment, reinforcing the notion that activation of p38MAPKα and p38MAPKβ in ESFT cells abolished PARP cleavage, a marker of fenretinide-induced death that is induced early in apoptosis. In contrast, inhibition of the delayed activation in SH-SY5Y cells had no effect on cell death or PARP cleavage. A recent study has described sustained activation of p38MAPK and JNK after exposure to fenretinide in the neuroblastoma cell lines KP-N-TK and KP-N-SIFA (47); however, the authors failed to show a causative link between activation of p38MAPK or JNK and induction of cell death. Furthermore, activation was only detected after exposure of cells to high concentrations of fenretinide (10 μmol/L) for 4 hours, consistent with the hypothesis that activation of these stress-activated kinases is an effector of fenretinide-induced death in neuroblastoma cells and not an important initiating event. JNK is also activated after 4-hour exposure of TC-32 cells to fenretinide, suggesting a role as an effector of cell death. JNK activation was unaltered following inhibition of p38MAPK but was abolished following inhibition of ROS accumulation, consistent with the hypothesis that JNK activation is a consequence of ROS accumulation and not p38MAPK signaling. Consequently, JNK may amplify the death response in a cell line–dependent mechanism through a parallel complimentary mechanism. However, this hypothesis requires further investigation. Our data show for the first time that ROS-dependent rapid and sustained activation of p38MAPK plays a fundamental role in fenretinide-induced cell death of ESFT cell lines and may contribute to the high sensitivity of these cells to fenretinide. The importance of p38MAPK is consistent with our recent observation that activation of this stress-activated kinase is a rate-limiting step in basic fibroblast growth factor–induced death of ESFT (27).

The potency with which fenretinide induces apoptosis of in vitro monolayer cell cultures and spheroids and the efficacy of fenretinide observed in vivo suggest that this retinamide may offer therapeutic benefits to ESFT patients. Further work is required to understand fully the mechanism by which fenretinide induces cell death in ESFT, although the data presented here suggest that ROS accumulation in response to fenretinide leads to the activation of p38MAPK (and later JNK), which may be responsible in part for the high sensitivity of ESFT cells to fenretinide (Fig. 7). However, it is clear that fenretinide may be a much needed drug for the successful treatment of ESFT, a disease for which there are few effective treatments. The next step will be to optimize and characterize the in vivo pharmacologic properties of fenretinide and to investigate interactions between fenretinide and conventional or novel chemotherapeutic agents in ESFT.

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p38MAPK-Dependent Sensitivity of Ewing's Sarcoma Family of Tumors to Fenretinide-Induced Cell Death

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