Medical and Pediatric Oncology 35:597–602 (2000)

Retinoic-Acid-Resistant Neuroblastoma Cell Lines Show Altered *MYC* Regulation and High Sensitivity to Fenretinide

C.P. Reynolds, MD, PhD,[1,2,3]* **Y. Wang,** PhD,[1,3] **L.J. Melton,** BA,[1]
P.A. Einhorn, MS,[1] **D.J. Slamon,** MD, PhD,[4] **and B.J. Maurer,** MD, PhD,[1,2]

Background. High-dose, pulse-13-*cis*-retinoic acid (13-*cis*-RA) given after intensive cytotoxic therapy improves event-free survival for high-risk neuroblastoma (NB), but more than 50% of patients have tumor recurrence. ***Procedure.*** We conducted multistep selection for resistance to all-trans-retinoic acid (ATRA) in NB cell lines with (SMS-KCNR and LA-N-5) or without (SMS-LHN) *MYCN* genomic amplification. ***Results.*** After 12 exposures to 10 μM ATRA, the two *MYCN*-amplified cell lines (KCNR 12X RR and LA-N-5 12X RR) showed partial resistance to the cytostatic/differentiation effects of ATRA; complete resistance was seen in LHN 12X RR. ATRA-selected cells showed general RA resistance (cross-resistance to 13-*cis*-RA). Transient (KCNR 12X RR, LA-N-5 12X RR) or sustained (LHN 12X RR) novel overexpression of *c-myc* was associated with RA resistance. RA-insensitive overexpression of MYCN by transduction in SMS-LHN also conferred RA resistance. Both parental and RA-resistant lines showed 2–4 logs of cell kill in response to *N*-(4-hydroxyphenyl)retinamide (4-HPR, fenretinide). Compared to parental lines, 4-HPR achieved 1–3 log greater cell kills in RA-resistant LHN 12X RR, LA-N-5 12X RR, KCNR 12X RR, and *MYCN*-transduced SMS-LHN or SK-N-RA. NB cell lines (n = 26) from 21 different patients showed that 16 of 26 (62%) were sensitive to 4-HPR (LC_{90} < 10 μM), including lines established at relapse after myeloablative and/or 13-*cis*-RA therapy. ***Conclusion.*** Thus, RA-resistant NB cell lines can be sensitive (and in some cases collaterally hypersensitive) to 4-HPR. Med. Pediatr. Oncol. 35:597–602, 2000. © 2000 Wiley-Liss, Inc.

Key words: neuroblastoma; retinoic acid; fenretinide; *MYCN;* c-*myc*

INTRODUCTION

Neuroblastoma is an aggressive childhood neoplasm of the sympathetic nervous system. Intensive chemoradiotherapy supported with bone marrow transplantation has improved survival for high-risk neuroblastoma, especially if followed by 13-*cis*-retinoic acid (13-*cis*-RA) [1]. Clinical use of 13-*cis*-RA, which achieves higher drug levels [2,3] than all-trans-retinoic acid (ATRA), was based on studies with neuroblastoma cell lines. Treatment of both *MYCN*-gene amplified and nonamplified human neuroblastoma cell lines with either of the retinoic acids (RA), ATRA or 13-*cis*-RA, caused a marked decrease in MYCN RNA expression and arrest of cell proliferation [4–6]. In some cell lines, 10 days of 5–10 μM RA treatment caused a prolonged growth arrest that persisted for >60 days after drug removal, and the growth arrest correlated with decreased MYCN RNA and protein expression [4]. The few cells that reproliferated after the sustained growth arrest responded again to 5–10 μM RA with decreased MYCN expression and arrested cell proliferation [4]. These data suggested that RA causes growth arrest of neuroblastoma by downregulation of MYCN expression and that RA resistance could be caused by a failure of MYCN downregulation. If the latter is true, new agents targeted at overcoming RA resistance would need either to downregulate MYCN expression via a mechanism that overcomes resistance to RA, or alternatively to achieve cytotoxicity and/or sustained growth arrest of neuroblastoma cells by other mechanisms.

N-(4-hydroxyphenyl)retinamide (fenretinide, 4-HPR) is a synthetic retinoid made in the late 1960s that has been reported to be cytotoxic for neuroblastoma cell lines [7–9] via generation of reactive oxygen species and ceramide [10]. Here we show that RA resistance in neuroblastoma cell lines is associated with a lack of downregulation of total myc (*MYCN* and/or c-*myc*) gene expression by RA. We also show that many neuroblastoma cell lines are sensitive to 4-HPR, and that neuroblastoma cell lines selected for resistance to RA are collaterally hypersensitive to 4-HPR.

[1]Division of Hematology-Oncology, Childrens Hospital Los Angeles, Los Angeles, California

[2]Department of Pediatrics, University of Southern California School of Medicine, Los Angeles, California

[3]Department of Pathology, University of Southern California, School of Medicine, Los Angeles, California

[4]UCLA School of Medicine, Los Angeles, California

*Correspondence to Dr. C. Patrick Reynolds, Division of Hematology-Oncology, MS#57, Childrens Hospital Los Angeles, 4650 Sunset Boulevard, Los Angeles, CA 90027. E-mail: preynolds@chla.usc.edu

© 2000 Wiley-Liss, Inc.

MATERIALS AND METHODS

Cell Lines

We studied 26 neuroblastoma cell lines [11–15]. SMS-SAN, SMS-LHN, SMS-KAN, SMS-KANR, SMS-KCNR, LA-N-5, LA-N-6, SK-N BE(2), SK-N-SH, SK-N-FI, SK-N-RA, LHN 12X RR, KCNR 12X, RR, and LA-N-5 12X RR were cultured in complete medium made from RPMI-1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gemini BioProducts, Inc., Calabasas, CA). CHLA-11, CHLA-15, CHLA-20, CHLA-51, CHLA-60, CHLA-70, CHLA-79, CHLA-90, CHLA-134, CHLA-136, CHLA-140, and CHLA-150 were cultured in complete medium made from Iscove's Modified Dulbecco's Medium (BioWhittaker, Walkersville, MD) supplemented with ≈3 mM L-glutamine (Gemini BioProducts, Inc.,) insulin and transferrin 5 μg/ml each, and 5 ng/ml of selenious acid (ITS Culture Supplement; Collaborative Biomedical Products, Bedford, MA) and 20% heat-inactivated FBS. Cell lines were free of mycoplasma and were subcultured using Pucks saline A + 1 mM EDTA [13].

Gene Expression and Transduction

Expression of MYCN and c-myc were measured using Northern and Western blotting as previously described [15,16]. A MYCN cDNA spanning exons 2 and 3 was inserted into the EcoR I site of the LXSN retroviral vector [17], which also contained the neo resistance gene. The LXSN retroviral vector alone (empty vector) or LXSN containing *MYCN* was used to infect the SMS-LHN and SK-N-RA cell lines and clones were selected using G418 (GibcoBRL Products, Gaithersberg, MD) [18].

Cytotoxicity Assay

Cytotoxicity of fenretinide for neuroblastoma cell lines was determined over a range of concentrations from 0.5 to 12 μM using the DIMSCAN assay system [19,20]. Cytotoxicity relative to vehicle-treated controls was measured 5 to 7 days after initiating drug exposure, depending on doubling time of cells being tested. LC_{90} values (i.e., the drug concentration that was cytotoxic for 90% of a cell population) were calculated using the software Dose-Effect Analysis with Microcomputers [11,12]. Cell lines with an LC_{90} value ≥10 μM were considered resistant to 4-HPR.

Apoptosis Assay

Apoptosis was detected by TUNEL assay, using the ApopTag kit (Oncor, Gaithersberg, MD). Cells were fixed in 1% paraformaldehyde, and terminal DNA fragments were identified by two-color flow cytometry using fluorescein-labeled anti-digoxigenin antibodies against digoxigenin-nucleotide conjugates that were catalytically added by terminal deoxynucleotidyl transferase; DNA counterstaining used propidium iodide [21]. Analysis was carried out on a Becton Dickinson FACScan flow cytometer with a 488 nM argon laser.

RESULTS

Resistance to RA and myc Expression

To explore the mechanism of RA resistance in neuroblastoma, we exposed SMS-LHN (*MYCN* nonamplified) LA-N-5, and SMS-KCNR (*MYCN* gene-amplified) to 10 μM ATRA for 10 days, allowed the cells to re-establish a proliferating cell population, and re-treated cells with 10 μM ATRA. We repeated these steps 12 times. Initial recovery of proliferating cells in SMS-LHN required > 60 days, with a faster recovery (~10 days) of proliferation after the second RA exposure, and no growth arrest observed with the fourth ATRA treatment, or with subsequent RA treatments. Only transient growth arrest (<10 days after removal of ATRA) was seen at any selection point for SMS-KCNR or LA-N-5. Cell lines resistant to ATRA were cross-resistant to 13-*cis*-RA and are considered generally RA resistant.

MYCN RNA and protein expression was only minimally downregulated in SMS-KCNR and LA-N-5 selected for RA resistance, with rapid recovery of MYCN expression after removal of ATRA. SMS-LHN showed diminished downregulation of MYCN after recovery from the first exposure to ATRA, but continued to show measurably decreased MYCN expression in response to ATRA. However, SMS-LHN RA-resistant cells showed overexpression of c-myc that was not downregulated by ATRA. After 12 exposures of SMS-LHN to ATRA (cells designated as LHN 12X RR), no MYCN expression was detectable, and very high expression of c-myc was observed. Transient expression of c-myc was seen in SMS-KCNR and LA-N-5 during selection for RA resistance, but c-myc expression was lost after several passages of RA-resistant cell lines in RA-free medium, accompanied by only minimal downregulation of MYCN expression if subsequently challenged with ATRA.

To determine whether a failure to downregulate MYCN expression was responsible for RA resistance in neuroblastoma, we transfected the *MYCN* gene into SMS-LHN so that it was expressed constitutively and not downregulated by ATRA. This was done using a cDNA for *MYCN* covering exons 2 and 3 that was inserted into the EcoR I site of the LXSN retroviral vector, with the Moloney murine leukemia virus (Mo-MuLV) long terminal repeats serving as a transcriptional regulator. SMS-LHN clones transduced with LXSN-*MYCN* expressed an exogenous 2.2 kb MYCN RNA and showed an approximately fivefold increase in MYCN protein by immunoblotting relative to parental SMS-LHN or empty vector

controls. MYCN expression in transduced cells showed no downregulation of MYCN RNA or protein when treated with 10 μM ATRA. LXSN (empty-vector)-transduced SMS-LHN showed MYCN downregulation by ATRA and sustained growth arrest (>20 days) after ATRA withdrawal, whereas *MYCN*-transduced SMS-LHN cells showed only transient growth arrest during RA exposure, and proliferated at the rate of control cells immediately after ATRA withdrawal. Thus, downregulation of MYCN is a key event in the induction of sustained growth arrest of neuroblastoma by ATRA.

Cytotoxicity of 4-HPR

We determined whether 4-HPR was active against neuroblastoma cell lines that were selected for resistance to RA. As shown in Figure 1, a high degree of apoptosis was seen in LHN 12X RR cells (93% in 4-HPR-treated cells versus 11% seen in control cells treated with ethanol vehicle only). The LHN 12X RR cells were resistant to growth inhibition by ATRA or 13-*cis*-RA (see above), and LHN 12X RR treated with ATRA showed 13% apoptotic cells, relative to 11% in the vehicle-treated control (Fig. 1).

We determined the activity of 4-HPR (using the DIMSCAN assay, which can measure up to 4 logs of cytotoxicity) against a panel of 23 neuroblastoma cell lines, which were established from 21 different patients after various phases of therapy, as well as the three cell lines selected in vitro for RA resistance. The cell line panel included the parental (preselection) cell lines of the RA-resistant lines (Table I). Of the 26 cell lines tested, 16 were sensitive to 4-HPR (LC_{90} values < 10 μM, range from < 0.5 to 9.2 μM), and 13 of 26 cell lines had an LC_{90} < 4 μM. Although 4-HPR resistance was common in those lines established at the time of progressive disease, some cell lines established after myeloablative therapy and/or 13-*cis*-RA therapy remained sensitive to 4-HPR.

All three cell lines selected in vitro for resistance to RA were collaterally hypersensitive to the cytotoxic effects of 4-HPR. In response to 3 μM 4-HPR, cell kills were 4 logs for LHN 12 X RR vs 1.5 logs for SMS-LHN; 2.5 logs for KCNR 12X RR vs 0.7 logs for SMS-KCNR; and 4 logs for LA-N-5 12X RR vs 2.5 logs for LA-N-5. Thus, all three RA-resistant cell lines showed decreased 4-HPR LC_{90} values relative to the non-RA-selected parental cell lines (Table I). A similar collateral hypersensitivity was seen when MYCN was overexpressed by transduction with LXSN in SMS-LHN or SK-N-RA. Both *MYCN*-transduced cell lines had a greater sensitivity to 4-HPR (LC_{90} < 0.5 μM for SMS- LHN; LC_{90} = 4.4 μM for SK-N-RA), compared to the parental cell lines (LC_{90} = 2.5 μM for SMS-LHN; LC_{90} > 15 μM for SK-N-RA), or the empty vector controls (LC_{90} = 1.75 μM for SMS-LHN; LC_{90} > 15 μM for SK-N-RA).

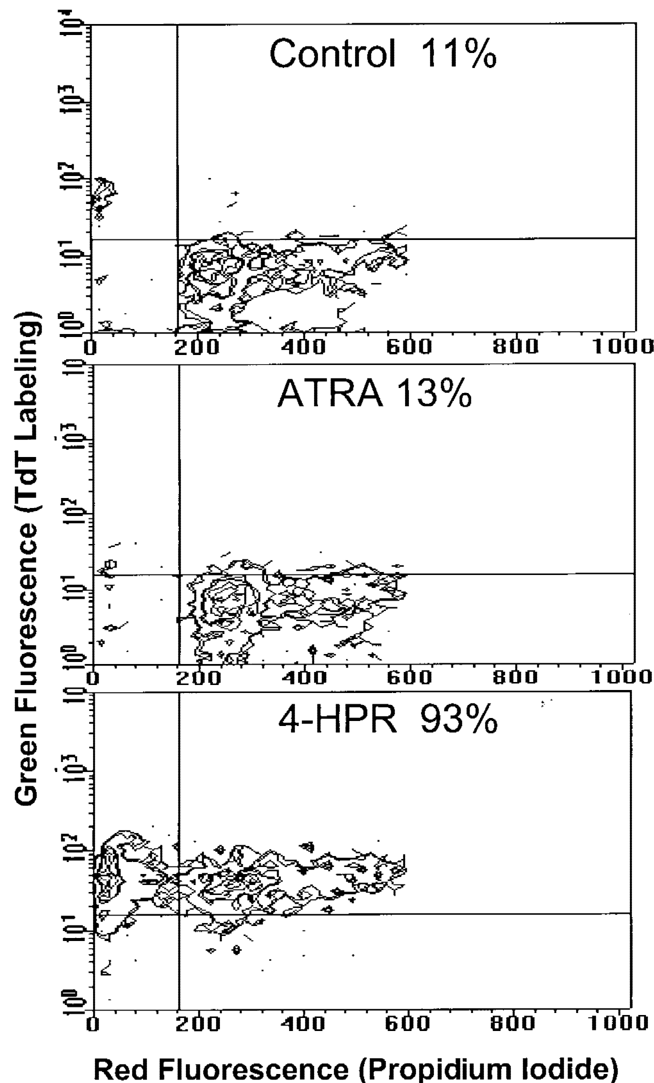

Fig. 1. Apoptosis was quantified by flow cytometry for the LHN 12X RR cell line (a retinoic-acid–resistant variant of SMS-LHN) using terminal deoxynucleotidyl transferase (TdT)-mediated labeling of DNA fragments with digoxigenin-dUTP followed by fluorescein-labeled antidigoxigenin (*Y* axis, green fluorescence) and DNA content by propidium iodide (PI) staining (*X* axis, red fluorescence). Gated quadrants and percent apoptic cells were based on staining of control (viable) LHN 12X RR cells with a nonbinding antibody and PI. **Top panel** shows control cells, treated with vehicle (ethanol) for 36 hs. **Middle panel** shows cells treated with 10 μM all-trans-retinoic acid (ATRA) for 36 hs. **Bottom panel** shows cells treated with 10 μM *N*-(4-hydroxyphenyl)retinamide (4-HPR) for 36 hs. Note that the majority of 4-HPR-treated cells show TdT labeling of DNA fragments. A portion of the cells showed a sub-G_0 DNA staining. Both are indicators of apoptosis.

DISCUSSION

The improvement in event-free survival seen in high-risk neuroblastoma patients treated with 13-*cis*-RA after

TABLE I. The 26 Cell Lines Studied for Sensitivity to *N*-(4-hydroxyphenyl)Retinamide (4-HPR) and the 4-HPR LC_{90} Values (in μM) for each*

Phase of therapy	Cell line	4-HPR LC_{90} (μM)
DX	CHLA-15	6.2
	SMS-KAN	2.3
	SMS-SAN	0.1
	LA-N-5	1.3
PD	CHLA-20	>15
	CHLA-60	9.2
	LA-N-6	1.9
	SK-N-BE(2)	15.7
	SK-N-SH	>15
	SK-N-FI	>15
	SK-N-RA	>15
	SMS-KANR	3.0
	SMS-KCNR	3.8
	SMS-LHN	2.5
PD-BMT	CHLA-51	6.1
	CHLA-79	>15
	CHLA-134	>15
	CHLA-136	14.0
Post 13-*cis*-RA	CHLA-70	>15
	CHLA-140	1.2
	CHLA-150	>10
Post PD-BMT + 13-*cis*-RA	CHLA-11	3.7
	CHLA-90	2.6
Selected for RA resistance	LHN 12X RR	0.03
	KCNR 12X RR	1.65
	LA-N-5 12X RR	0.05

*Lines in the panel included those established at diagnosis before therapy (DX), at time of progressive disease during or after nonmyeloablative therapy (PD), after myeloablative therapy (PD-BMT), after therapy with 13-*cis*-retinoic acid (Post 13-*cis*-RA), and after myeloablative therapy followed by 13-*cis*-RA (Post PD-BMT + 13-*cis*-RA). Also shown are the three cell lines (LHN 12X RR, KCNR 12X RR, and LA-N-5 12X RR) selected for RA resistance from the RA-sensitive cell lines, SMS-LHN, SMS-KCNR, and LA-N-5. 4-HPR LC_{90} values were derived from dose–response curves using DIMSCAN to measure the cytotoxic response.

completion of cytotoxic therapy [1] suggests that further improvements in outcome can be achieved by developing agents effective against minimal residual disease (MRD) that are tolerable soon after completion of myeloablative therapy [22]. However, to be successful, new therapies for MRD in neuroblastoma must be effective against tumor cells resistant to 13-*cis*-RA. We have approached the development of such new agents by the in vitro selection of RA-resistant cell lines from *MYCN*-amplified and nonamplified lines that were initially RA sensitive. We have also established a panel of cell lines from patients who developed progressive disease after various therapies, including myeloablative and/or 13-*cis*-RA therapy.

Neuroblastoma cell lines selected for RA resistance appear to mediate resistance primarily by failing to downregulate myc expression. In the SMS-LHN cell line, which lacks genomic amplification of *MYCN,* we observed that selection for RA resistance initially resulted in a diminished downregulation of MYCN expression and a shorter period of arrested cell proliferation. On the fourth and subsequent ATRA exposures, we observed no arrest of proliferation, yet there was still MYCN downregulation; however, the RA-resistant SMS-LHN cells showed novel overexpression of c-myc. Further RA selection led to a complete replacement of MYCN expression by c-myc in SMS-LHN, even after removal of ATRA.

The two *MYCN*-amplified lines (LA-N-5 and SMS-KCNR) also showed increased c-myc expression when selected for RA resistance, but this effect was transient. Presumably the c-myc expression was replaced (in terms of total myc expression) by the development of high-level MYCN expression that showed minimal downregulation by ATRA after several exposures. It is likely that the ability of *MYCN*-amplified cell lines to develop RA-insensitive MYCN expression was facilitated by the multiple copies of the *MYCN* genome and associated promoters that are present in these cell lines. The role of non-RA-downregulated myc expression in conferring RA resistance was confirmed by transduction of an RA-insensitive *MYCN* cDNA, which conferred RA resistance in SMS-LHN. Taken together, these data imply that RA-insensitive myc expression (MYCN and/or c-myc) is required by neuroblastomas to overcome the antiproliferative effects of RA.

Fenretinide (4-HPR) has been reported to be cytotoxic for neuroblastoma cell lines in vitro at 1–10 μM concentrations in a dose-dependent manner [7–9]. In contrast to 13-*cis*-RA and ATRA, 4-HPR does not induce maturational changes, but is cytotoxic, causing both apoptosis [7,23–25] and necrosis [10,26]. 4-HPR has shown activity against non-neuroblastoma cell lines known to be resistant to ATRA [27–30], suggesting that it could have activity against RA-resistant neuroblastomas.

We found that not only were those cell lines selected for RA resistance sensitive to 4-HPR, but they were also collaterally hypersensitive, showing a higher degree of cell kill and lower 4-HPR LC_{90} values than did the nonselected cell lines. In one of these cell lines (LHN 12X RR), we observed a high degree of apoptosis induced by 4-HPR, suggesting that an increased apoptotic response to 4-HPR may be one mechanism of collateral hypersensitivity in the RA-resistant cell lines. Because all three RA-resistant cell lines have a high degree of total myc expression (MYCN or c-myc), and because increasing myc expression by *MYCN*-transduction also increased 4-HPR sensitivity, the level of total myc expression may relate to the sensitivity of neuroblastoma cells to 4-HPR-mediated apoptosis. This would be consistent with the observation that 4-HPR sensitivity in small-cell lung can-

cer cell lines related to c-myc expression [24]. However, 4-HPR also mediates death via a p53- and caspase-independent pathway (especially at concentrations ~ 10 μM), which causes necrosis rather than apoptosis, likely via production of large amounts of ceramide [10]. Studies in progress are addressing the relationship of myc expression to 4-HPR sensitivity.

Toxicity of 4-HPR in chemoprevention clinical trials (with chronic low dosing) has been minimal and no hematologic toxicity has been reported, with the major clinical toxicity of 4-HPR being decreased night vision because of decreased plasma retinol levels [31]. A similar lack of toxicity has been seen with pulse (1 week of 4-HPR, 2 weeks rest) dose escalation in a pediatric phase I trial, which is achieving plasma levels > 8 μM, with dose escalation still in progress [32]. We have found that 16 of the 26 cell lines tested had a 4-HPR LC_{90} value within the clinically achievable range for the drug, including 3 of the 5 cell lines that were established at time of relapse after 13-*cis*-RA therapy. This, combined with the collateral hypersensitivity demonstrated in three cell lines selected for RA resistance in vitro, suggests that following 13-*cis*-RA therapy with 4-HPR could decrease tumor recurrence. Therapy with 4-HPR could be especially effective if used against MRD that has been selected for RA resistance because such tumor cells may have collateral hypersensitivity to 4-HPR.

ACKNOWLEDGMENTS

Supported in part by the Neil Bogart Memorial Laboratories of the T. J. Martell Foundation for Leukemia, Cancer, and AIDS Research; by National Cancer Institute grant CA81403; and by a grant from the American Institute for Cancer Research.

REFERENCES

1. Matthay K, Villablanca JG, Seeger RC, et al. Treatment of high risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. N Engl J Med 1999;341:1165–1173.
2. Khan AA, Villablanca JG, Reynolds CP, et al. Pharmacokinetic studies of 13-cis-retinoic acid in pediatric patients with neuroblastoma following bone marrow transplantation. Cancer Chemother Pharmacol 1996;39:34–41.
3. Villablanca JG, Khan AA, Avramis VI, et al. Phase I trial of 13-cis-retinoic acid in children with neuroblastoma following bone marrow transplantation. J Clin Oncol 1995;13:894–901.
4. Reynolds CP, Kane DJ, Einhorn PA, et al. Response of neuroblastoma to retinoic acid in vitro and in vivo. Prog Clin Biol Res 1991;366:203–211.
5. Reynolds CP, Schindler PF, Jones DM, et al. Comparison of 13-cis-retinoic acid to trans-retinoic acid using human neuroblastoma cell lines. Prog Clin Biol Res 1994;385:237–244.
6. Thiele CJ, Reynolds CP, Israel MA. Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. Nature 1985;313:404–406.
7. Di Vinci A, Geido E, Infusini E, et al. Neuroblastoma cell apoptosis induced by the synthetic retinoid N-(4-hydroxyphenyl)-retinamide. Int J Cancer 1994;59:422–426.
8. Mariotti A, Marcora E, Bunone G, et al. N-(4-hydroxyphenyl)-retinamide: a potent inducer of apoptosis in human neuroblastoma cells. J Natl Cancer Inst 1994;86:1245–1247.
9. Ponzoni M, Bocca P, Chiesa V, et al. Differential effects of N-(4-hydroxyphenyl)retinamide and retinoic acid on neuroblastoma cells: apoptosis versus differentiation. Cancer Res 1995;55:853–861.
10. Maurer BJ, Metelitsa LS, Seeger RC, et al. N-(4-hydroxyphenyl)-retinamide increases ceramide and reactive oxygen species and induces mixed apoptosis/necrosis in neuroblastoma cell lines. J Natl Cancer Inst 1999;91:1138–1146.
11. Keshelava N, Groshen S, Reynolds CP, Cross-resistance of topoisomerase I and II inhibitors in neuroblastoma cell lines. Cancer Chemother Pharmacol 2000;45:1–8.
12. Keshelava N, Seeger RC, Groshen S, et al. Drug resistance patterns of human neuroblastoma cell lines derived from patients at different phases of therapy. Cancer Res 1998;58:5396–5405.
13. Reynolds CP, Biedler JL, Spengler BA, et al. Characterization of human neuroblastoma cell lines established before and after therapy. J Natl Cancer Inst 1986;76:375–387.
14. Reynolds CP, Tomayko MM, Donner L, et al. Biological classification of cell lines derived from human extra-cranial neural tumors. Prog Clin Biol Res 1988;271:291–306.
15. Wada RK, Seeger RC, Brodeur GM, et al. N-*myc* expression in human neuroblastoma cell lines without N-*myc* gene amplification. Cancer 1993;72:3346–3354.
16. Wang Y, Einhorn P, Triche TJ, et al. Expression of protein gene product 9 5 and tyrosine hydroxylase in childhood small round cell tumors. Clin Cancer Res 2000;6:551–558.
17. Peng HJ, Einhorn PA, Wong S, et al. N-myc transduction into human neuroblastoma cells enhances aggressive behavior. Proc Am Assoc Cancer Res 1995;36:185.
18. Mohn A, Koller BH. Retroviral vectors. In: DNA *cloning: a practical approach.* Oxford: IRL Press. 1995. p 113–142.
19. Fragala T, Proffitt RT, Reynolds CP. A novel 96-well plate cytotoxicity assay based on fluorescence digital imaging microscopy. Proc Am Assoc Cancer Res 1995;36:303.
20. Proffitt RT, Tran JV, Reynolds CP. A fluorescence digital image microscopy system for quantifying relative cell numbers in tissue culture plates. Cytometry 1996;24:204–213.
21. Li X, James WM, Traganos F, et al. Application of biotin, digoxigenin or fluorescein conjugated deoxynucleotides to label DNA strand breaks for analysis of cell proliferation and apoptosis using flow cytometry. Biotech Histochem 1995;70:234–242.
22. Reynolds CP, Lie SO. Retinoid therapy of neuroblastoma. In: Brodeur GM, Sawada T, Tsuchida Y, Voute PA, editors. *Neuroblastoma.* Amsterdam: Elsevier; 2000. p 519–540.
23. Dipietrantonio A, Hsieh TC, Wu JM. Differential effects of retinoic acid (RA) and N-(4-hydroxyphenyl) retinamide (4-HPR) on cell growth, induction of differentiation, and changes in p34cdc2, Bcl-2, and actin expression in the human promyelocytic HL-60 leukemic cells. Biochem Biophys Res Commun 1996;224:837–842.
24. Kalemkerian GP, Slusher R, Ramalingam S, et al. Growth inhibition and induction of apoptosis by fenretinide in small-cell lung cancer cell lines [see comments]. J Natl Cancer Inst 1995;87: 1674–1680.
25. Ziv Y, Gupta MK, Milsom JW, et al. The effect of tamoxifen and fenretinimide on human colorectal cancer cell lines in vitro. Anticancer Res 1994;14:2005–2009.
26. Clifford JL, Menter DG, Wang M, et al. Retinoid receptor-

dependent and-independent effects of N-(4-hydroxyphenyl)retinamide in F9 embryonal carcinoma cells. Cancer Res 1999;59:14–18.
27. Delia D, Aiello A, Lombardi L, et al. N-(4-hydroxyphenyl)retinamide induces apoptosis of malignant hemopoietic cell lines including those unresponsive to retinoic acid Cancer Res 1993;53: 6036–6041.
28. Kazmi SM, Plante RK, Visconti V, et al. Comparison of N-(4-hydroxyphenyl)retinamide and all-trans-retinoic acid in the regulation of retinoid receptor-mediated gene expression in human breast cancer cell lines. Cancer Res 1996;56:1056–1062.
29. Sheikh MS, Shao ZM, Li XS, et al. N-(4-hydroxyphenyl)retinamide (4-HPR)-mediated biological actions involve retinoid receptor-independent pathways in human breast carcinoma. Carcinogenesis 1995;16:2477–2486.
30. Supino R, Crosti M, Clenci M, et al. Induction of apoptosis by fenretinide (4HPR) in human ovarian carcinoma cells and its association with retinoic acid receptor expression. Int J Cancer 1996;65:491–497.
31. Torrisi R, Parodi S, Fontana V, et al. Factors affecting plasma retinol decline during long-term administration of the synthetic retinoid fenretinide in breast cancer patients. Cancer Epidemiol Biomarkers Prev 1994;3:507–510.
32. Bagniewski PG, Reid JM, Villablanca JG, et al. A phase I pharmacokinetic study of fenretinide (HPR) in children with high-risk solid tumors. Proc Am Assoc Cancer Res 1999;40:92.