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Antitumor activity of sodium valproate in cultures of human neuroblastoma cells

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Valproic acid (VPA) is a simple branched-chain fatty acid that has anticonvulsant activity and is widely used in the treatment of epilepsy. VPA was found to effect growth and differentiation of human neuroblastoma (NB) cells *in vitro* at concentrations that have been achieved in humans with no significant adverse effects. Treatment of UKF-NB-2 and UKF-NB-3 NB cell lines with VPA at concentrations ranging from 0.5 to 2 mM resulted in neuronal morphological differentiation characterized by extension of cellular processes without significant effects on cell viability. Ultrastructural features of VPA-treated cells were consistent with the neuronal type of differentiation. VPA treatment of NB cells was associated with decreased expression of N-*myc* oncoprotein and increased expression of neural cell adhesion molecule in their membrane. Treatment of NB cells with 0.5 mM VPA increased their sensitivity to lymphokine-activated killer lysis. The results indicate that VPA, at non-toxic pharmacological concentrations, arrests the growth, induces differentiation and increases immunogenicity of NB cells through non-toxic mechanisms.

Key words: Cell differentiation, lymphokine-activated killer cells, neural cell adhesion mole-cule, neuroblastoma, sodium valproate.

Introduction

Neuroblastoma (NB) is a common pediatric malignant neoplasm with a poor prognosis in the presence of disseminated disease or loss of cellular differentiation. The ability of NB cells to differentiate *in vitro* and *in vivo* suggests the possibility of new therapeutic approaches using differentiation-inducing agents. Several of these agents have been used in the treatment of patients with hematopoietic and solid tumors, including retinoids, sodium butyrate, interferons, 5-azacytidine, hexamethylene bisacetamide and sodium phenylacetate.[1–3] The clinical application of some agents has been limited by toxicities such as potential carcinogenesis, and inability to achieve and sustain effective plasma concentrations. The potential value of differentiation induction therapy has been proven by the management of acute promyelocytic leukemia using all-*trans*-retinoic acid (ATRA).[3] RA has been shown to induce differentiation, growth arrest and decreased expression of the N-*myc* oncogene in human NB cell lines *in vitro*.[4] Not all NB cell lines respond to RA; NB cells grown in the continuous presence of RA may develop resistance to retinoids.[5] It is possible that such factors may contribute to the limited clinical benefit of RA observed in several patients suffering from NB.[4]

There is considerable interest in short-chain fatty acids such as *n*-butyric acid (BA) and its derivatives as candidates for differentiation therapy. However, clinical reports dealing with the treatment of leukemia are not encouraging.[3] The lack of clinical effects is probably due to the fact that infused BA is rapidly metabolized and that the plasma concentrations are below the concentrations in the millimolar range that are generally needed for effects *in vitro*. During infusion, the plasma concentration of BA increased about 6-fold over the endogenous level to 39–59 μM and a plasma half-life of only 6 min was seen after infusion was stopped.[3] Butyryl triglyceride (tributyrine or glyceryl tributyrate) is a candidate prodrug for butyrate that can be administrated orally. Tributyrine was shown to induce differentiation of human leukemic HL-60 cells and on a molar basis it was 4-fold more potent than BA.[6] Aromatic fatty acids including phenylacetate and its prodrug phenylbutyrate have a broad antiproliferative and differentiation

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activity in various hematopoietic and solid tumors.[7–11] Clinical experience obtained during phenylacetate treatment of cancer and of urea cycle disorders indicates that phenylacetate concentrations producing significant antitumor effects *in vitro* can be achieved in humans without significant adverse effects.[12,13] Since the aromatic ring resists β-oxidation, phenylacetate and phenylbutyrate are relatively stable substances with a plasma half-life of 3 and 1–2 h, respectively.[12,14]

Valproic acid (di-*n*-propylacetic acid, VPA) is a short-chain fatty acid, branched in contrast to BA, that has broad spectrum anticonvulsant activity and is used in the treatment of epilepsy.[15] VPA has been tested for its ability to stimulate production of fetal hemoglobin in individuals with β-hemoglobinopathies.[16] VPA shows mild to moderate toxic effects at clinically effective doses in most patients. Only small numbers of patients develop serious side effects such as liver damage.[15] VPA has a significantly longer plasma half-life (9–16 h) than BA or other short-chain fatty acids yet tested in clinical trials.[15] Therapeutic regimens using oral formulations of VPA are well established.[15] These properties of VPA encouraged us to investigate whether VPA may be regarded as a therapeutic agent for human malignancies. In the present study we tested the effects of VPA on growth, differentiation, expression of cell adhesion molecules (CAMs) and sensitivity to natural killer/lymphokine-activated killer (NK/LAK) lysis of two NB cell lines *in vitro*.

Materials and methods

Cell lines and reagents

NB cell lines UKF-NB-2 and UKF-NB-3 were established from metastasis harvested in relapse in two of our patients with Evans stage 4 neuroblastoma.[10,17] All culture media and media supplements were purchased from Seromed (Berlin, Germany). The cells were propagated in IMDM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. Normal embryonal cells including human embryonal lung fibroblasts (HEL) and human amnion epithelial cells (HA) were established and grown as described previously.[17] VPA was obtained as the sodium salt from Sigma (Deisenhofen, Germany). VPA was dissolved in IMDM and stored in aliquots at 4°C. ATRA was obtained from Sigma and dissolved in dimethyl sulfoxide.

Assessment of cell growth

Cells growing in logarithmic phase were seeded in polystyrene culture flasks at a density 1×10^4 cells/cm^2 and incubated in a culture medium without or with VPA. On day 8 of treatment, cells were harvested with 0.2% trypsin and the cell number was determined using a hemocytometer. Cell viability was determined by Trypan blue exclusion.

Characterization of NB cell differentiation

NB cells were seeded at a density 2×10^3 cells/cm^2 and incubated without or with VPA. The cultures were examined daily with a phase-contrast microscope for assessment of morphological changes. Ultrastructural features of NB cultures incubated without or with VPA were examined using transmission electron microscopy. The cells processed for ultrastructural analysis were pelleted and fixed with 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in ethanol and embedded in Durupan-Epon. Thin sections were contrasted with uranyl acetate and lead citrate, and viewed with a Joel, JEM, 200 CX microscope.

Western blot analysis

Western blot analysis for N-*myc* oncoprotein in extracts obtained from UKF-NB-3 cells incubated for 8 days without or with VPA was performed as described previously.[18] Samples prepared from NB cells were fractionated by electrophoresis on a denaturating sodium dodecyl sulfate–7.5% polyacrylamide gel under reducing conditions. Fractionated proteins were transferred electrophoretically to nitrocellulose membranes. N-*myc* was detected using mouse mAb (Ab-1; Dianova, Hamburg, Germany) which recognizes nuclear phosphoproteins of 64 and 67 kDa.

Measurement of cell surface CAMs

For quantitative analysis of the expression of cell surface CAMs, UKF-NB-2 and UKF-NB-3 cell lines were incubated for 8 days without or with different concentrations of VPA or RA. The expression of neural cell adhesion molecule (NCAM), intercellular adhesion molecule-1 (ICAM-1), endothelial leukocyte adhesion molecule-1 (ELAM-1), vascular cell adhe-

sion molecule-1 (VCAM-1) and lymphocyte function associated-3 (LFA-3) was measured by means of flow cytometry (FACscan; Becton Dickinson, Heidelberg, Germany). ICAM-1, ELAM-1 and VCAM-1 were marked by mouse mAb (clone BBIG-I1, BBIG-E6 and BBIG-V1, respectively; all Bierman, Bad Nauheim, Germany). LFA-3 and NCAM were detected using mAb clone BRIC5 (Camon, Wiesbaden, Germany) and mAb clone NCAM-OB11 (Sigma), respectively. NB cells stained with primary antibodies were treated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 1 h at 4°C. Intensity of surface molecule expression was detected and evaluated by means of Lysis II software.

Cytotoxicity assay

The capability of NK and LAK cells to lyse NB cells as target cells was determined by means of a fluorescence-based europium release assay described in detail elsewhere.[19] Human peripheral blood mononuclear cells (PBMC), isolated from the blood of healthy volunteers by Ficoll-Hypaque centrifugation, were used as a source of NK and LAK effector cells. Freshly isolated PBMC were incubated overnight to allow the adherence of monocytes to the bottom of culture flasks. The cell suspension was removed the next day from the flasks and used as a source of NK effector cells. To generate LAK effector cells, non-adherent PBMC were further treated with 200 U/ml interleukin (IL)-2 for 5 days. The measurement of europium release without effector cells served as negative control (spontaneous release), target cells lysed with 0.5% Triton X-100 as positive control (maximum release); 5×10^4 target cells/ml were incubated with different quantities of effector cells to obtain the final effector to target (E:T) ratios 40:1, 20:1, 10:1 and 5:1. All NK and LAK experiments were performed at least three times, using different blood donors.

Results

Effects of VPA on cell proliferation

Proliferation of UKF-NB-2 and UKF-NB-3 cell lines was inhibited by VPA at concentrations ranging from 0.25 to 2 mM in a dose-dependent manner (Figure 1). These effects were similar in both cell lines with IC_{50} values of 0.51 and 0.65 mM for UKF-NB-2 and UKF-NB-3 cell lines, respectively. NB cultures treated with VPA at concentrations up to 2 mM showed cell viability comparable to that of untreated control cultures. Concentrations greater than 2 mM were significantly toxic for both NB cell lines. Proliferation of normal human cell types including HEL and HA was inhibited to a significantly lower extent than that of NB cells (Figure 1); IC_{50} for HEL and HA cells was 5.1 and 4.6 mM, respectively. Viability of HEL and HA cells was not influenced by treatment with VPA at concentrations up to 8 mM.

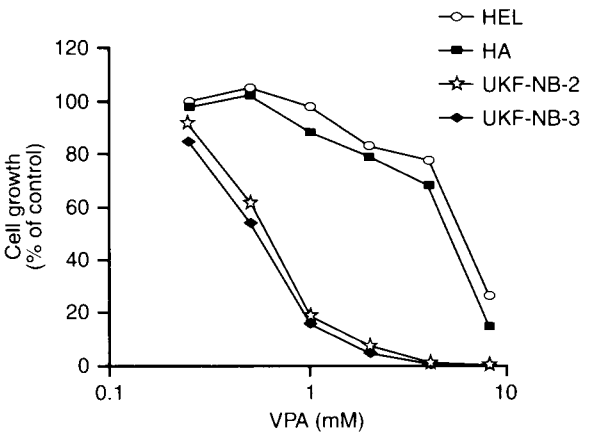

Figure 1. Effects of VPA on cell proliferation of UKF-NB-2, UKF-NB-3, HA and HEL cell lines. The cells were incubated for 8 days without or with different concentrations of VPA. Point, mean ($n = 4$).

Effects of VPA on cell differentiation

Morphological differentiation was induced in both NB cell lines after treatment with non-toxic concentrations of VPA ranging from 0.5 to 2 mM. Figure 2 depicts cultures treated for 16 days with 0.5 mM VPA showing extension of neurits. Maximum differentiation was observed in cultures treated with 2 mM VPA. Ultrastructural examination of UKF-NB-3 cells treated with VPA confirmed neuronal-type differentiation. Untreated UKF-NB-3 cultures consisted mostly of cells that did not show features of neuroblastic differentiation (Figure 3A). UKF-NB-3 cultures treated for 16 days with 0.5 mM VPA exhibited tightly packed cells forming specialized membrane contacts resembling desmosomes (Fig. 3B and 3C). The cytoplasma of treated cells contained numerous dense-core vesicles and significantly higher numbers of mitochondria than untreated cells (Figure 3B). Neuritic processes of VPA-treated cells contained microtubules (Figure 3D) as well as dense-core vesicles and numerous organelles (Figure 3E).

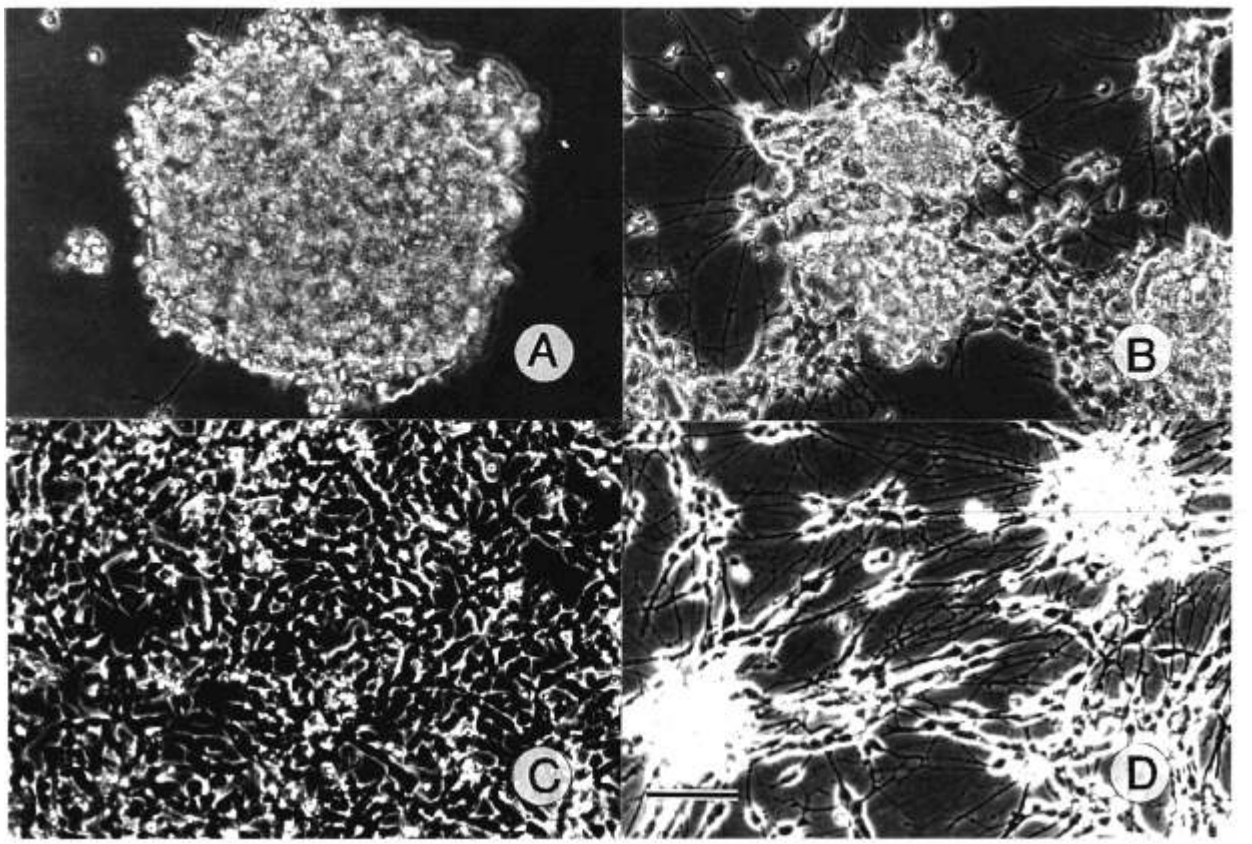

Figure 2. Morphological appearance of the human NB cell lines UKF-NB-2 and UKF-NB-3 in the absence and presence of VPA. UKF-NB-2 (A) and UKF-NB-3 (C) cells treated for 16 days with solvent control showed the morphology of poorly differentiated neuroblastic cells. UKF-NB-2 (B) and UKF-NB-3 (D) cells treated for 16 days with 0.5 mM VPA showed morphologic differentiation characterized by extension of neurits. Bar represents 80 μm.

Effects of VPA on N-*myc* expression

Expression of the N-*myc* oncoprotein has been shown to be decreased in UKF-NB-3 cells treated for 8 days with 0.5 and 1 mM VPA (Figure 4). Scanning densitometry measurements (of different experiments) showed that 2- to 3-fold and 4- to 6-fold less N-*myc* oncoprotein accumulated in cells treated with 0.5 and 1 mM VPA than in untreated control cultures, respectively (data not shown).

Effects of VPA on expression of CAMs

In order to evaluate the influence of VPA on surface CAMs, expression of ICAM-1, ELAM-1, VCAM-1, LFA3 and NCAM was quantified by flow cytometry. Treatment of both UKF-NB-2 and UKF-NB-3 cell lines with VPA at concentrations of 0.5 and 1 mM augmented basal expression of NCAM (Figure 5) but not other CAMs tested (data not shown). Incubation of NB cell lines with 1 μM RA had no effect on NCAM expression (Figure 5).

Sensitivity of UKF-NB-2 and UKF-NB-3 cells to NK and LAK lysis

To investigate the putative role of NCAM in MHC-unrestricted cell-mediated anti-tumor responses, we tested whether increased expression of NCAM stimulated by VPA treatment may be associated with enhanced sensitivity of NB cell lines to NK and LAK lysis. UKF-NB-2 and UKF-NB-3 cells were resistant to NK lysis which was not influenced by VPA treatment (data not shown). In contrast, both cell lines were sensitive to LAK cell killing (Figure 6). As a high variability of LAK activity occurs among different normal blood donors, results shown in Figure 6 represent one experiment (repeated at least three

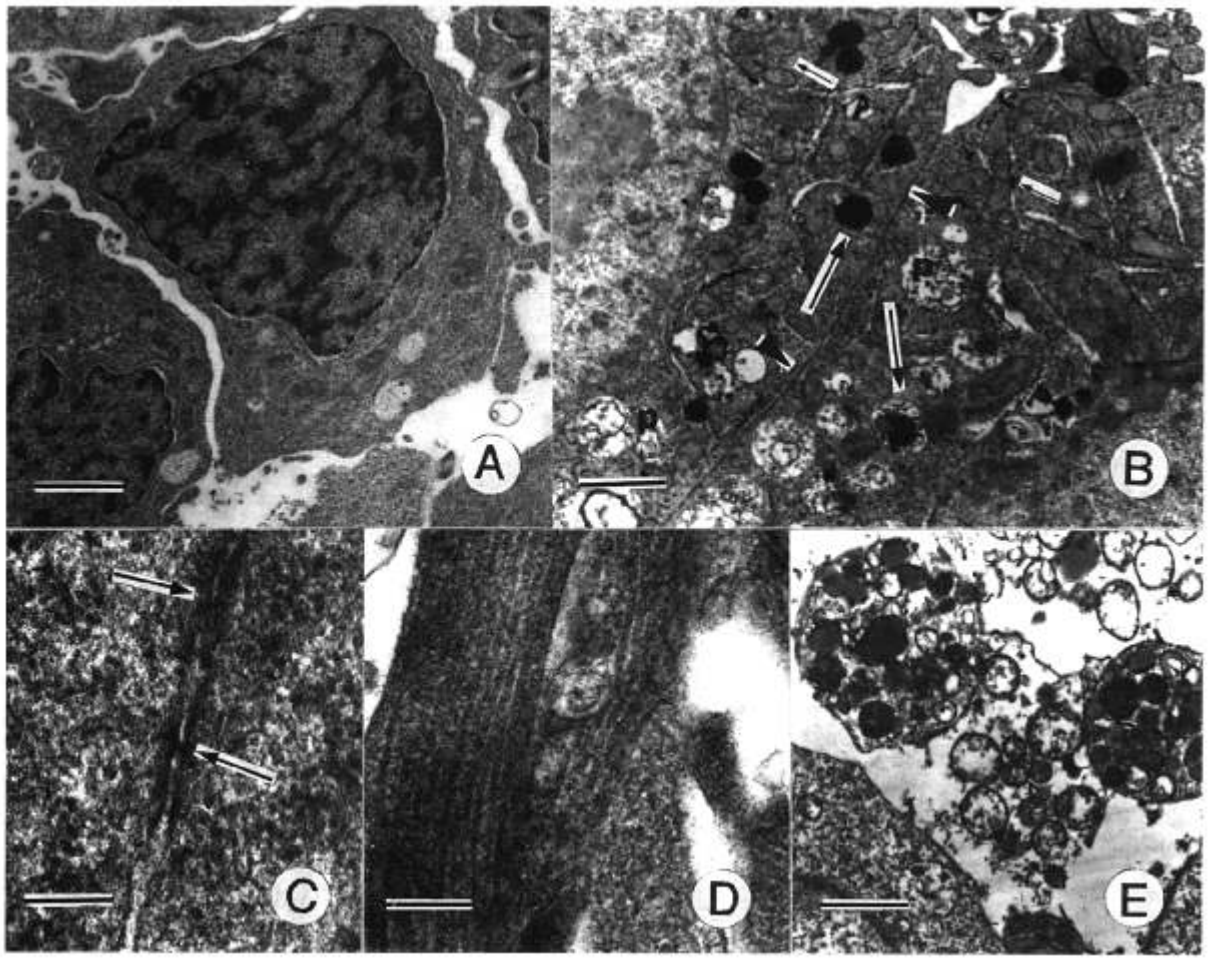

Figure 3. Ultrastructural features of UKF-NB-3 cells in the absence and presence of VPA. Untreated UKF-NB-3 cultures contained mostly cells without features of neuroblastic differentiation (A). UKF-NB-3 cultures treated for 16 days with 0.5 mM VPA contained in the cytoplasma (p) numerous dense-core vesicles (large arrows) and high numbers of mitochondria (small arrows) (B). Neighboring cells of VPA-treated cultures formed specialized membrane contacts (arrowheads) (B) resembling desmosomes (arrows) (C). Neuritic processes contained microtubules (D); on the cross-section of neuritic processes numerous dense-core vesicles and organelles were observed (E). Bars represent 2 μm (A), 1 μm (B), 430 nm (C), 300 nm (D) and 1.4 μm (E).

times). The data demonstrate that UKF-NB-3 cells were more sensitive to LAK than UKF-NB-2 cells; LAK at an E:T ratio of 40:1 lysed 8 and 13% of UKF-NB-2 and UKF-NB-3 cells, respectively. The treatment of both NB cell lines with VPA at a concentration of 0.5 mM increased sensitivity to lysis; LAK lysis at an E:T ratio of 40:1 was increased 3- and 4-fold for UKF-NB-2 and UKF-NB-3 cells, respectively. The treatment of NB cell lines with ATRA did not increase sensitivity to NK/LAK lysis (data not shown).

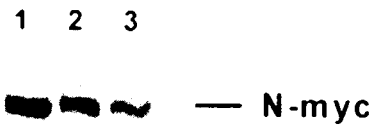

Figure 4. Effects of VPA on N-*myc* oncoprotein expression in UKF-NB-3 cell line. Western blot analysis of N-*myc* was performed in cells incubated for 8 days without VPA (lane 1) and with 0.5 mM (lane 2) or 1 mM (lane 3) VPA.

Discussion

We have demonstrated that VPA induces neuronal differentiation of the cultured NB cells at pharmacologically attainable concentrations as assessed by dose-dependent growth inhibition, neurite outgrowth, ultrastructural features characteristic of neuronal cells and decreased expression of N-*myc*

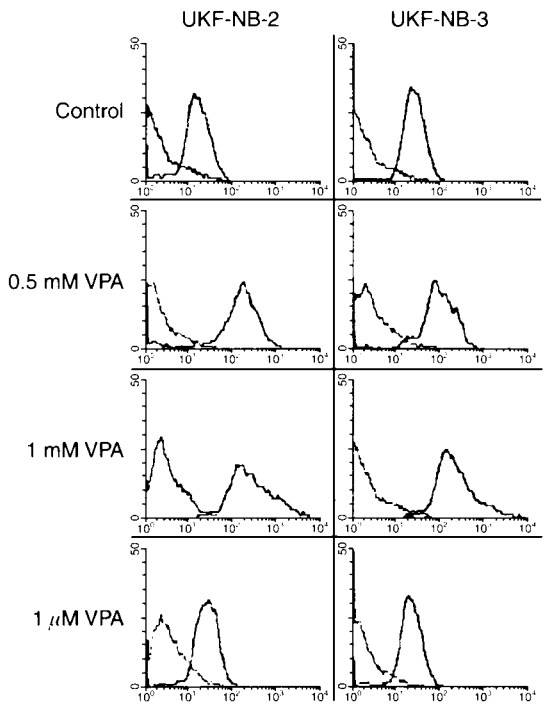

Figure 5. Effects of VPA and ATRA on expression of surface NCAM in NB cell lines. Quantitative analysis of surface NCAM was performed in UKF-NB-2 and UKF-NB- 3 cells treated for 8 days with 0.5 or 1 mM VPA and 1 μM ATRA. NCAM-specific fluorescence intensities are depicted as histograms (right curves) and compared with unspecific signals obtained with an irrelevant mAb (left curves).

oncoprotein. VPA treatment was accompanied by increased surface expression of NCAM and increased sensitivity of NB cells to LAK lysis. These results suggest that in addition to direct antitumor effects on NB cells, VPA could be of importance as a drug increasing immunogenicity of tumor cells.

VPA is a short-chain fatty acid, with biological properties similar to other structurally related substances. Indeed, differentiation activity on human NB cells is prominent for VPA and other short-chain fatty acids such as sodium butyrate and aromatic fatty acids (i.e. phenylbutyrate and phenylacetate). Despite a large number of reports on the modulation of gene expression by short chain-fatty acids, little is known of their mechanism(s) of action. The aromatic fatty acids and VPA can conjugate with coenzyme A, enter the pathway to chain elongation and interfere with lipid metabolism in general.[20,21] One of the possible mechanisms could result from effects on protein prenylation. The aromatic fatty acids were shown to inhibit cholesterol synthesis (possibly due to their resemblance to mevalonic acid) and prenylation of $p21^{ras}$, and possibly other proteins which are involved in the regulation of cell growth and differentiation.[11,22] Another mechanism could stem from effects of short-chain fatty acids on DNA methylation. Both butyrate and aromatic fatty acids were shown to influence DNA methylation and consequently the expression of methylation-dependent genes.[8,23] It remains to be elucidated if some of these effects account for antitumor activity of VPA on NB cells.

VPA treatment increased expression of surface NCAM in UKF-NB-2 and UKF-NB-3 cell lines while expression of other adhesion molecules including ICAM-1, VCAM-1, ELAM-1 and LFA-3 was not significantly influenced. Treatment with RA did not influence NCAM expression in UKF-NB-2 and UKF-NB-3 cells even though these NB cells differentiate during RA treatment.[10] In the previous studies RA treatment did not effect NCAM expression in SH-IN cell line,[24] while treatment of SK-N-BE cell line was associated with increased expression of surface NCAM.[25] Preliminary data show that VPA stimulates monocytic differentiation of leukemic HL-60 cells in association with increased NCAM expression while granulocytic differentiation of HL-60 cells induced by RA does not result in increased NCAM expression (unpublished results). These results suggest that VPA may be a common stimulator of NCAM expression independently of a neuronal phenotype of cells.

NCAM (Leu-19 or CD56) is expressed by all NK cells and a subpopulation of T lymphocytes. NCAM-mediated homotypic adhesion between target and effector cells has been proposed to participate in the sensitivity of NB cells to NK/LAK cytolysis.[26] UKF-NB-2 and UKF-NB-3 cells were not sensitive to NK lysis; and VPA treatment did not induce their sensitivity to NK cells. Contrasting this, both NB cell lines were sensitive to LAK lysis which was increased by VPA treatment. *In vivo* studies suggest that NK cell cytotoxicity in children with NB is enhanced by treatment with IL-2.[27] However, such treatments revealed severe toxicities resulting from the use of IL-2. Treatment strategies using combinations with other cytokines such as IL-12 were studied with the aim to improve LAK-based immunotherapy of NB.[28] Alternatively, strategies may be developed to increase sensitivity of a tumor target to NK/LAK lysis. In the previous studies induction of differentiation in NB cell lines with differentiation agents including RA, interferon-γ, phorbol ester or 5'-bromodeoxyuridine did not increase sensitivity of NB cells to NK/LAK lysis.[24] VPA is a unique differentiation inducer in terms of its ability to increase sensitivity of NB cells to LAK lysis.

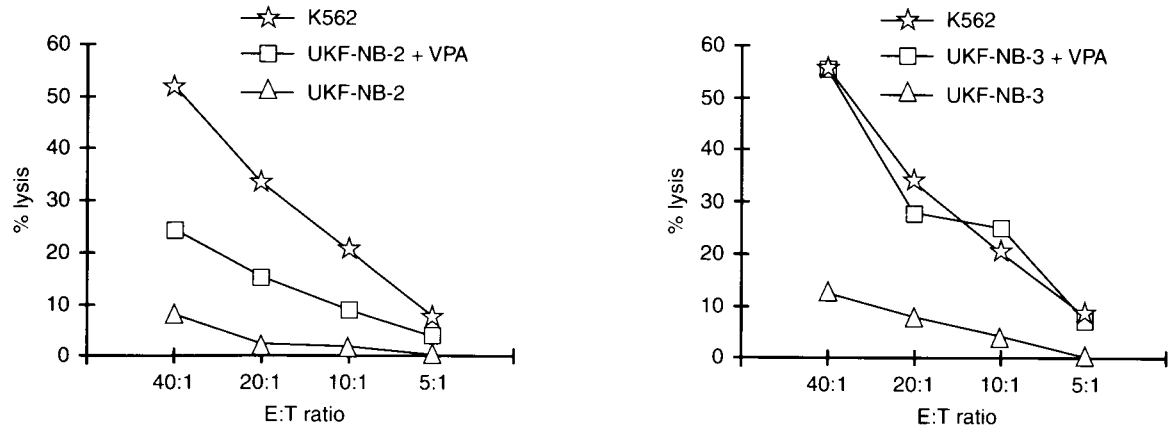

Figure 6. Effects of VPA on the sensitivity of neuroblastoma cell lines to LAK lysis. Sensitivity to LAK cell killing was measured in UKF-NB-2 and UKF-NB-3 cell lines treated for 8 days with 0.5 mM VPA. Point, mean ($n = 3$).

Therapeutic plasma VPA concentrations for most patients treated for epilepsy will range from 0.24 to 0.6 mM (40–100 mg/l).[15] However, there is not a clear-cut relation among the plasma concentrations of VPA, its anticonvulsive effects and toxicity. Some patients need and can tolerate plasma concentrations as high as 0.9 mM (150 mg/l). The most common adverse effects associated with VPA are mild to moderate in severity. VPA-induced fatal hepatotoxicity occurs in less than 1 in 20000 patients. There is an estimated risk of 1–2% for neural tube defects in newborns of mothers treated with VPA during pregnancy.[15] In patients with β-hemoglobinopathies VPA induces higher levels of fetal hemoglobin (one to two orders on a molar basis) than other short-chain fatty acids.[16] This may be at least in part due to a significantly longer plasma half-life of VPA (9–16 h) when compared with those of other related substances such as BA (6 min), phenylbutyrate (1–2 h) or phenylacetate (3 h).

In conclusion, we have shown that VPA at pharmacological concentrations that are well tolerated by patients with epilepsy exhibit high antitumor activity against NB cells *in vitro*. As pharmacokinetic and pharmacodynamic data are much better than those observed with other short-chain fatty acids proposed for the treatment of patients with cancer we suggest to extend the use of VPA to cancer patients for prevention and therapy.

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