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Sodium valproate inhibits *in vivo* growth of human neuroblastoma cells

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Sodium valproate (VPA) belongs to the group of simple branched-chain fatty acids and due its anticonvulsive activity is broadly applied in the treatment of epilepsy. We previously showed that VPA is able to induce cellular differentiation, to enhance immunogenicity and to inhibit proliferation of human neuroblastoma (NB) cells *in vitro*. Furthermore, we demonstrated that VPA inhibits proliferation, enhances neural cell adhesion molecule expression and decreases CD44 expression of human and rat glioma cells *in vitro*. In the present study we investigated the antitumoral effects of VPA on established human NB xenografts from UKF-NB-3 human NB cells in athymic (nude) mice. When the animals developed s.c. tumors of about 100 mm^3 volume they were treated with 400 or 200 mg/kg/day VPA i.p. At the end of the treatment period (40 days) tumor volumes in animals treated with 400 and 200 mg/kg VPA were about 4- ($p < 0.0001$) and 2-fold ($p < 0.0005$) smaller than in the saline-treated control group, respectively. Histological examination of the remnant tumors of treated animals revealed induction of differentiation by induction of stroma-rich tumors and nodules that contained elongated NB cells. Pyknotic nuclei and apoptotic bodies indicated induction of apoptosis. We conclude that VPA is able to abrogate NB growth *in vivo* and may therefore be useful in the treatment of NB patients.

Key words: Apoptosis, cell differentiation, neuroblastoma, sodium valproate, xenograft.

Introduction

Neuroblastoma (NB) is the most common solid malignant tumor outside the central nervous system in childhood. As many patients are diagnosed with disseminated disease, prognosis remains poor despite the introduction of high-dose chemotherapy with stem cell rescue. Unfortunately even patients with favorable prognostic factors such as lacking 1p36 deletion and N-*myc* amplification may develop progressive disease.[1–4] NB cells are derived from elements of the neural crest and are able to differentiate *in vitro* and *in vivo* spontaneously or when treated with agents such as short-chain fatty acids including phenylacetate and butyrate, retinoids, interferons and 5-azacytidine as well as cytosine arabinoside.[5–10] This observation is the fundamental basis for alternative clinical therapies aiming at the induction of differentiation and inhibition of proliferation to control aggressive NB growth. Until now the clinical benefit of these substances in the treatment of NB has been restricted due to potential carcinogenesis, low effective plasma levels or cellular chemoresistance.[11,12] Therefore it remains necessary to search for other anti-NB agents.

Aromatic fatty acids including phenylacetate and its prodrug phenylbutyrate possess antitumoral properties and are able to induce differentiation in several solid and hematopoetic malignancies.[7,13–15] These effects have been observed *in vitro* and *in vivo* at concentrations that had been achieved earlier in patients treated with phenylacetate for urea cycle disorders without adverse side effects.[7,13–16] Their clinical antitumoral application has been limited in part by a short plasma half-life of 3 h.[12] In contrast valproic acid (di-*n*-propylacetic acid, VPA) is a branched-chain fatty acid and has a significantly longer half-life of 9–16 h.[17] There is broad clinical experience of VPA treatment in adult and pediatric patients with different forms of epilepsy. Short- and long-term oral treatment is sometimes accompanied by mild side effects. Only few patients (1 in 20 000) have been affected by serious toxic effects such as liver failure.[18] However, clinical investigations have shown that VPA treatment significantly elevated HbF in patients with-

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out β-hemoglobinopathies.[19] In previous studies we were able to show that *in vivo* treatment of NB cell lines UKF-NB-2 and UKF-NB-3 with VPA at concentrations clinically used against epilepsy ranging from 0.2 to 1.5 mM resulted in inhibition of cellular proliferation and neuronal morphological differentiation characterized by extension of cellular processes which was confirmed by ultrastructural studies.[6] The viability of the cells was not affected. Treated NB cells showed decreased expression of N-*myc* oncoprotein and increased expression of neural cell adhesion molecule (NCAM, CD56) on their cell surface whereas the expression of intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1, CD106), endothelial leukocyte adhesion molecule-1 (ELAM-1) and lymphocyte function associated-3 (LFA-3) remained unchanged. These phenomena were associated with an enhanced sensitivity of VPA-treated NB cells to lymphokine activated killer cell-induced lysis.[6] In additional studies we demonstrated that other cells of neuroectodermal origin, i.e. human and rat glioma cells, were also growth arrested in a dose-dependent manner by the same concentrations as NB cells when treated with VPA. Furthermore, VPA-treated glioma cells expressed less hyaluronic acid receptor (CD44) and more NCAM on their cell membrane.[20] Encouraged by these strong antitumoral effects on NB and glioma cells *in vitro* we studied the efficacy of VPA in the treatment of human NB xenografts in athymic nude mice.

Material and methods

Cells and reagents

Human NB cell line UKF-NB-3 was established from metastasis harvested in relapse in one of our patients with Evans stage 4.[21,22] Culture media and 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 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. VPA was obtained as sodium salt from Sigma (Deisenhofen, Germany). VPA was dissolved in phosphate-buffered saline and stored in aliquots at −20°C.

Mice

Seven-week-old female CD-1 nude mice were obtained from Charles River nude mice colony (Charles River, AnLab, Czech Republic) 1 week before each experiment. Mice were housed in a barrier facility in sterile isolated cages. All manipulations of cages and animals were conducted in a laminar air-flow room. Animals received a standard sterile diet.

Subcutaneous xenografts of UKF-NB-3

Antitumoral activity of VPA was determined in CD-1 nude mice, bearing established s.c. UKF-NB-3 xenografts. For this, UKF-NB-3 tumors grown in maintenance mice were removed under sterile conditions, and passed through a series of screens and needles of decreasing bore. After that 0.05 ml of the tumor cell suspension was implanted into the right flank of mice. As the median size of the tumors reached about 100 mm^3 (day 0 of treatment) animals were divided into three groups of eight: control group (average tumor volume 102 mm^3) and two treatment groups (average tumor volume 107 and 99 mm^3) as to establish an equal distribution of average tumor size between all three groups. One treatment group received VPA i.p. (0.2 ml/dose) at a daily dose of 400 mg/kg whereas the second treatment group received 200 mg/kg. Control animals received an equal volume of vehicle (saline) by the same schedule. To assess the effects of VPA, tumors were measured in two dimensions with a Vernier caliper and tumor volume was calculated using the formula V=length × width^2/2, where length is the longer of the two measurements.[23] Significance of tumor growth inhibition was estimated by Student's t-test.

Histopathological examination of s.c. xenografts of UKF-NB-3

Tissues for histology wre fixed in 4% buffered formalin, embedded in paraffin and sectioned at 4 μm. All sections were stained with hematoxylin & eosin and examined for pathological findings by light microscopy.

Results

Effects of *in vivo* treatment on UKF-NB-3 growth

To measure the effects of VPA on the growth of UKF-NB-3 xenografts, tumor-bearing animals were treated with a daily i.p. dose of 400 or 200 mg/kg. In previous

experiments, these VPA concentrations did not induce any toxic effects in treated animals.[5] In concert with previous studies there was no observable toxicity in the animals treated with VPA. After 13 and 25 days of treatment, tumor volumes in the animals treated with 400 or 200 mg/kg VPA were significantly smaller than in control animals, respectively ($p < 0.005$ for both observations) (Figure 1). Final tumor volumes (after 40 days of treatment) were about 4- and 2-fold smaller in animals treated with 400 or 200 mg/kg VPA than in control animals, respectively (1430 ± 135 mm^3 for the control group; 365 ± 54 mm^3 for the group treated with 400 mg/kg VPA, $p < 0.0001$; 687 ± 69 mm^3 for the group treated with 200 mg/kg VPA, $p < 0.0005$).

Histopathological examination of treated UKF-NB-3 tumors

Histological evaluations were performed on tumor tissues excised from animals treated for 40 days with VPA (400 mg/kg) and compared with tumor tissues from control animals treated with saline. The control tumors showed the features of classical stroma poor, undifferentiated NB (Figure 2A). Schwannian (stromal) development was absent. Fibrovascular septae were inconspicuous and there was no evident growth pattern of the tumor cells. The tumor was highly cellular with dense hyperchromatic nuclei and scanty cytoplasm. Homer-Wright rosettes were not detected and multiple mitoses were seen all over the tumor section. There were no hints of differentiating neuroblasts. Atypical and neoplastic mitoses that indicate a highly proliferating tumor cell population were frequently observed (Figure 2C). On the sections of treated tumors signs of differentiation in terms of a stroma-rich NB were readily evident (Figure 2B). There was a strong stromal component and organization into fields of highly cellular regions. These regions appeared to a certain extent nodular; the cells and nuclei were of enhanced density. The stromal parts showed prominent fibrillary features and contained low numbers of cells. These cells were predominantly elongated. Some tumor cells showed pyknotic nuclei that indicate apoptotic processes (Figure 2D). The nuclei showed an hyperdense nuclear membrane and were encircled by a halo. Within the nuclei several types of apoptotic bodies could be seen.

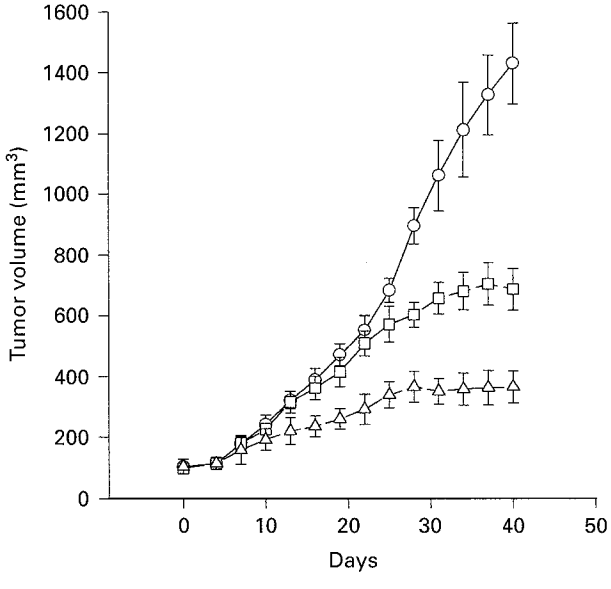

Figure 1. Inhibition of s.c. UKF-NB-3 xenografts in nude mice treated i.p. with 200 (□) and 400 (△) mg/kg/day VPA compared to controls (○). Points, mean values of eight experiments; bars, SEM.

Discussion

The data of the present study demonstrate for the first time that VPA has antitumoral effects on NB cells *in vivo*. We observed a significant growth inhibition of NB xenografts in nude athymic mice at non-toxic levels. This inhibition of NB growth was dose dependent and accompanied by an induction of NB cell differentiation and apoptotic features as shown by histopathological examination. These results are in accordance with our previous investigations that showed antitumoral activity of VPA in different cells of neuroectodermal origin *in vitro* such as NB cells as well as human and rat malignant glioma cells.[6,20] The dose dependence which is evident by a stronger tumor growth inhibition of NB xenograft tumors at 400 mg/kg VPA daily in comparison with 200 mg/kg VPA is consistent with the *in vitro* studies of NB and glioma cell treatment with VPA cells.[6,20] In our *in vitro* studies NB cells were triggered to differentiate towards mature neuronal cells, which was proven by ultrastructural studies.[6] The present results show that the remnant VPA-treated NB xenograft tumors exhibited prominent signs of differentiation at the histopathological evaluation. Untreated tumors showed no pattern or structure at all, whereas VPA-treated tumors were organized into stroma-rich and stroma-poor regions. The strong stromal component had lower cell density with elongated cell bodies. These histopathological features are consistent with a favorable histology as graded by the Shimada classification. This is supported by the fact that VPA treatment of NB cells *in vitro* leads to a strong reduction of N-*myc* oncoprotein, which is also closely related with a favorable NB

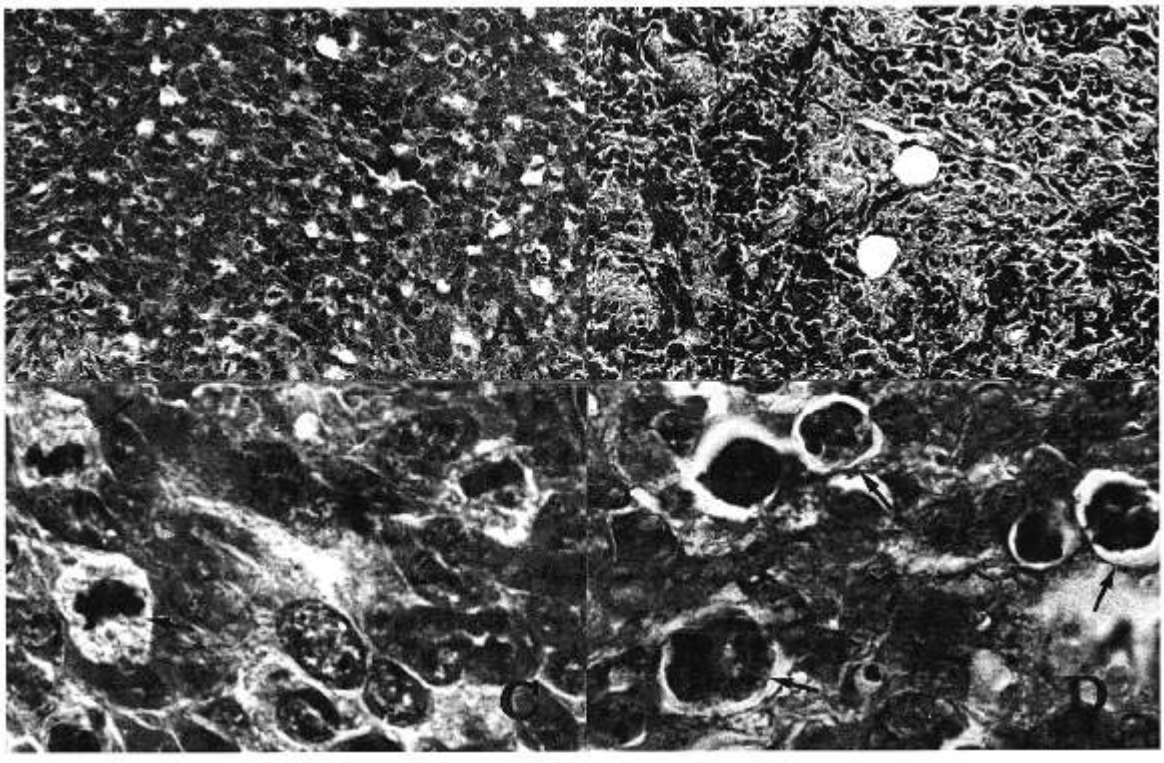

Figure 2. Histopathological examination of s.c. UKF-NB-3 xenografts. (A) Section of untreated tumor showing classical stroma poor undifferentiated NB (magnification × 67); note (C) atypical and neoplastic mitoses (arrows) indicating a highly proliferating cell population (magnification × 400). (B) Section of tumor treated with VPA (400 mg/kg) showing signs of differentiation in terms of stroma development (magnification × 67); note (D) numerous cells with picnotic nuclei and apoptotic bodies (arrows) (magnifications × 288); hematoxylin & eosin staining.

histology.[1,3,4] Nevertheless, Homer-Wright rosettes were inconspicuous in both treated and untreated xenograft tumors. Interestingly, cell viability in our *in vitro* studies was not affected by VPA treatment cells.[6,20] In contrast VPA-treated tumors showed signs of pyknotic and apoptotic bodies containing nuclei, indicating the induction of apoptosis in NB cells. The *in vitro* results of VPA treatment on neuroectodermal cells were accompanied by a change of cell surface molecule expression. Glioma and NB cells showed an elevation of NCAM (CD56) expressing cells.[6,20] Furthermore, in glioma cells CD44 expression (hyaluronic acid receptor) was lowered *in vitro* by VPA treatment cells.[20] It remains to be elucidated to what extent these phenomena may play a role in the growth inhibition of NB xenografts by VPA in nude mice.

Other studies investigating the *in vivo* antitumoral *in vivo* efficacy of retinoic acid and interferon-γ on NB xenografts in nude mice did not consistently show such strong inhibition of tumor formation. Additionally the remnant tumors of the treated animal groups did not show the prominent signs of differentiation and apoptosis that were present in the tumors treated with VPA.[5,8]

The mechanisms of the antitumoral activity of VPA have not been investigated yet. VPA belongs to the group of short-chain fatty acids with biological properties that are similar to other compounds of related structure. As shown in this *in vivo* study and our previous *in vitro* studies, VPA shares differentiation-inducing activity with other short-chain fatty acids such as phenylacetate, phenylbutyrate and sodium butyrate. The precise mechanism of action is still unknown but one mode of action may be explained by interference with lipid metabolism in general through modulation of chain elongation as VPA and other aromatic fatty acids can conjugate coenzyme A.[24,25] Another possibility could result from effects on protein prenylation as VPA and other fatty acids due to their resemblance interfere with the metabolic pathway of mevalonic acid. Thus, cholesterol synthesis and prenylation of $p21^{ras}$ and even other proteins involved in the regulation of cell growth and differentiation may be inhibited.[13,26] Other methods of antitumoral activity may result from effects of short-chain fatty acids on DNA methylation. These substances were shown to influence DNA methylation and hence the expression of methylation-dependent genes.[14,27]

Whether these effects are relevant for the antitumoral action of VPA on neuroectodermal cells remains to be elucidated.

To summarize, we have shown that VPA has strong antitumoral effects on NB cells *in vivo* which extends our previous findings of the *in vitro* antineoplastic efficacy of VPA on neuroectodermal tumor cells. These *in vivo* effects were reached at non-toxic levels in accordance with our *in vitro* results showing inhibition of cellular proliferation, induction of differentiation and enhancement of immunogenicity at pharmacological levels that are normally achieved in patients suffering from epilepsy. The favorable pharmacokinetic and pharmacodynamic data of VPA in comparison with other short-chain fatty acids makes it an attractive agent in the treatment of NB patients in addition to or after conventional chemotherapy to prevent metastasis or to control residual disease.

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