Valproic acid inhibits adhesion of vincristine- and cisplatin-resistant neuroblastoma tumour cells to endothelium

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Drug resistance to chemotherapy is often associated with increased malignancy in neuroblastoma (NB). In pursuit of alternative treatments for chemoresistant tumour cells, we tested the response of multidrug-resistant SKNSH and of vincristine (VCR)-, doxorubicin (DOX)-, or cisplatin (CDDP)-resistant UKF-NB-2, UKF-NB-3 or UKF-NB-6 NB tumour cell lines to valproic acid (VPA), a differentiation inducer currently in clinical trials. Drug resistance caused elevated NB adhesion (UKF-NB-2VCR, UKF-NB-2DOX, UKF-NB-2CDDP, UKF-NB-3VCR, UKF-NB-3CDDP, UKF-NB-6VCR, UKF-NB-6CDDP) to an endothelial cell monolayer, accompanied by downregulation of the adhesion receptor neural cell adhesion molecule (NCAM). Based on the UKF-NB-3 model, N-myc proteins were enhanced in UKF-NB-3VCR and UKF-NB-3CDDP, compared to the drug naïve controls. p73 was diminished, whereas the p73 isoform deltaNp73 was upregulated in UKF-NB-3VCR and UKF-NB-3CDDP. Valproic acid blocked adhesion of UKF-NB-3VCR and UKF-NB-3CDDP, but not of UKF-NB-3DOX, and induced the upregulation of NCAM surface expression, NCAM protein content and NCAM coding mRNA. Valproic acid diminished N-myc and enhanced p73 protein level, coupled with downregulation of deltaNp73 in UKF-NB-3VCR and UKF-NB-3CDDP. Valproic acid also reverted enhanced adhesion properties of drug-resistant UKF-NB-2, UKF-NB-6 and SKNSH cells, and therefore may provide an alternative approach to the treatment of drug-resistant NB by blocking invasive processes.

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Multiple-agent chemotherapy is the conventional therapy for patients with advanced stages of neuroblastoma (NB) and disseminated NB. However, drug resistance arises in the majority of stage IV and relapsed NB, often leading to treatment failure (Keshelava et al, 1998). Development of novel antitumoural strategies is therefore highly desired to overcome resistance mechanisms and to prevent tumour progression. Molecules modulating cellular function have been identified in the majority of tumours and their manipulation might be the key to decreasing malignancy.

Histone deacetylases (HDAC) represent one of the most important intracellular targets, as these molecules modulate a wide variety of cellular functions. Abnormal histone acetylation status can result in undesirable phenotypic changes, including developmental disorders and cancer. Indeed, aberrant histone acetylation may be an aetiologic factor in several types of cancer by derepressing gene transcription. Hence, HDAC inhibitors may be useful for cancer prevention, due to their ability to ‘reactivate’ the expression of epigenetically silenced genes, including those involved in differentiation, invasion and metastasis. Most notably, recent data indicate that HDAC inhibition may be successful in treating refractory or relapsing tumours after conventional chemotherapy. Histone deacetylation inhibition has been demonstrated to block cell growth of drug-resistant small-cell lung cancer lines (Tsurtani et al, 2003), abrogate resistance in breast cancer cells (Hirokawa et al, 2005) and induce apoptosis in drug-resistant ovarian cancer cells (Sonnemann et al, 2006), myeloma cells (Maiso et al, 2006) and hepatoma cell lines (Pathil et al, 2006). The branched-chain fatty acid valproic acid (VPA) has been shown to possess HDAC inhibitory properties and to affect the growth and survival of tumour cells in vitro and in vivo (Cinatl et al, 1996; Blaheta et al, 2005). This is highly relevant since VPA is an established drug in the long-term therapy of epilepsy. It can be applied orally, negative side effects are rare and it demonstrates expedient pharmacokinetic properties. To clearly assess whether VPA might be of benefit in treating relapsed NB, we evaluated the potential of therapeutic VPA concentration to block the interaction of drug-resistant NB cells with vascular endothelium. The experiments were based on an in vitro model of acquired drug resistance (Kotchekov et al, 2003, 2005), closely resembling progressive NB disease through long-term treatment of NB cell lines with vincristine (VCR), cisplatin (CDDP) or doxorubicin (DOX) to establish the resistant tumour cell sublines UKF-NB-3VCR, UKF-NB-3CDDP and UKF-NB-3DOX. A co-culture binding assay allowed the analysis of NB cells that adhered to an endothelial cell monolayer. Since surface receptors are strongly involved in tumour invasion and data have indicated that changes in neural cell adhesion molecule (NCAM, CD56) expression play an essential part in the progression of NB, we investigated NCAM expression (Blaheta et al, 2002), and the NCAM regulating proteins N-myc, p73 and deltaNp73 (Blaheta et al, 2004) under the influence of VPA.

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MATERIALS AND METHODS

Cell cultures and induction of drug resistance

The N-myc amplified human NB cell lines UKF-NB-2, UKF-NB-3 and UKF-NB-6 were established in our laboratory from bone marrow metastases. The CDDP-, VCR- and DOX-resistant UKF-NB-2, UKF-NB-3 and UKF-NB-6 sublines were established by exposing the parental cells to increasing concentrations of the drugs. Solutions of CDDP (Gry-Pharma, Kirchzarten, Germany), VCR (Sigma-Aldrich, Deisenhofen, Germany) and DOX (Farmitalia, Milan, Italy) were prepared in accordance to the manufacturer's instructions. The initial CDDP, VCR and DOX concentrations were 50, 0.2 and 2 ng ml\(^{-1}\) medium, respectively. The resistant sublines were grown for more than 6 months in Iscove's modified Dulbecco's medium (Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS, Gibco) and either 10 ng ml\(^{-1}\) VCR (UKF-NB-2\(^{\text{VCR}}\), UKF-NB-3\(^{\text{VCR}}\), UKF-NB-6\(^{\text{VCR}}\)), 20 ng ml\(^{-1}\) DOX (UKF-NB-2\(^{\text{DOX}}\), UKF-NB-3\(^{\text{DOX}}\), UKF-NB-6\(^{\text{DOX}}\), or 1000 ng ml\(^{-1}\) CDDP (UKF-NB-2\(^{\text{CDDP}}\), UKF-NB-3\(^{\text{CDDP}}\), UKF-NB-6\(^{\text{CDDP}}\)). The clinical relevance of drug-resistant UKF-NB-2 and UKF-NB-3 sublines has been examined before in a xenograft setting (Kotchekov et al., 2003, 2005). Multidrug resistant, not N-myc amplified SKNSH, were derived from LGC Promochem, Wesel, Germany. Cells were subcultured at 5-day intervals.

Chemoresistance of the indicated cell lines was verified by the MTT test (Kotchekov et al., 2003). The resistant cell lines showed at least 20-fold increase in resistance to the drugs, expressed as IC\(_{50}\).

Human endothelial cells (HUVEC) were isolated from human umbilical veins and harvested by enzymatic treatment with C.

VPA treatment

Parental NB tumour cells and their drug-resistant sublines were treated with VPA (gift from GL Pharma GmbH, Lannach, Austria) at a final concentration of 1 mM for 3 or 5 days. Tumour cell adhesion, NCAM, p73, deltaNp73 and N-myc expression were then measured in VPA-treated cells. Results were compared to untreated controls. Viability of tumour cells in presence of VPA was assessed by propidium iodide dsDNA intercalation.

Tumour cell adhesion

Human endothelial cells were transferred to six-well multiplates (Falcon Primaria; Becton Dickinson, Heidelberg, Germany) in complete HUVEC medium. When confluence was reached, 0.5 × 10\(^{6}\) parental NB tumour cells or their drug-resistant sublines (VPA-treated vs non-treated) were carefully added to the HUVEC monolayer for 60 min. Subsequently, non-adherent tumour cells were washed off using warmed (37°C) M199. The adherent cells were fixed with 1% glutaraldehyde and counted in five different fields (5 × 0.25 mm\(^2\)) using a phase contrast microscope (20 × objective) to calculate the mean cellular adhesion rate.

Cell proliferation

Proliferative activity of NB tumour cells and HUVEC was estimated by the PicoGreen assay as described elsewhere (Blaheta et al., 1998). Briefly, at several time points after plating the cells in six-well multiplates, culture medium was removed and cells were digested with papain (0.125 mg protein ml\(^{-1}\)) for 20 h at 60°C. Thereafter, the fluorescent dye PicoGreen (MoBiTec, Gottingen, Germany), which shows high specificity for dsDNA, was added (1:200 dilution) for 10 min at 20°C. Fluorescence intensity was determined using a computer-controlled fluorescence reader (Cytolux 2300 plate scanner; Millipore, Eschborn, Germany) at \(\lambda_{ex} = 485\) nm and \(\lambda_{em} = 530\) nm.

Evaluation of NCAM surface expression

Neuroblastoma cells were disaggregated mechanically, washed in blocking solution (PBS, 0.5% BSA) and then incubated for 60 min at 4°C with an FITC-conjugated monoclonal antibody anti-CD56 which detects the NCAM 120, 140 and 180 kDa isoform (clone 16.2). Neural cell adhesion molecule expression of NB cells was then measured using a FACSscan (Becton Dickinson; FL-1 H (log) channel histogram analysis; 1 × 10\(^4\) cells/scan) and expressed as mean fluorescence units (MFU). A mouse IgG1-FITC was used as the isotype control.

To explore NCAM localisation, tumour cells were transferred to round cover slips, which were placed in a 24-well multplate. Upon reaching confluence, cell cultures were washed two times with PBS (with Ca\(^{2+}\) and Mg\(^{2+}\)) and then fixed in cold (−20°C) methanol/acetone (60/40 v/v). Subsequently, cells were washed again with PBS (without Ca\(^{2+}\) and Mg\(^{2+}\)). The washing buffer, cells were incubated for 60 min with FITC-conjugated anti-NCAM monoclonal antibody. To prevent photo-bleaching of the fluorescent dye, cover glasses with stained cells were taken out of the wells and the residual liquid was removed. The cells were then embedded in an antifade reagent/mounting medium mixture (ProLong™ Antifade Kit, MoBiTec) and mounted on slides. The slides were viewed using a confocal laser-scanning microscope (LSM 10; Zeiss, Jena, Germany) with a plan-neofluar 100/1.3 oil immersion objective.

Western blot analysis

Total NCAM content in NB cells was evaluated by Western blot analysis: tumour cell lysates were applied to a 7% polyacrylamide gel and electrophoresed for 90 min at 60 V. The protein was then transferred to nitrocellulose membranes. After blocking, the membranes were incubated overnight with the anti-NCAM antibody (dilution 1:1000). HRP-conjugated goat anti-mouse IgG (Upstate Biotechnology, Lake Placid, NY, USA; dilution 1:5000) served as the secondary antibody. The membrane was briefly incubated with ECL detection reagent (ECL™, Amersham/GE Healthcare, München, Germany) to visualise the proteins and exposed to an x-ray film (Hyperfilm™ EC™, Amersham).

Semi-quantitative reverse transcription/PCRmerase chain reaction

Total RNA was extracted and purified with Trizol reagent according to the manufacturer’s instructions and treated with RNase-free DNase. The NCAM primer sequences were as follows: for NCAM-180: 5′ CGAGGCTGCCCTGCTGACAAG 3′ and 5′ CCGG ATCCATCATGCTGGTCTG 3′; for NCAM-140: 5′ GAAAGGATTCAACAGGATAGCGG 3′ and 5′ CGGATCATGCTGGTCTG 3′; for NCAM-104: 5′ GACACTTGGTCTGAGCT 3′. Ribonucleic acid (1–10 µg) was reverse transcribed and the resulting cDNA directly added to the PCR. Amplification reactions (20 µl) were performed in the presence of 1/10 (2 µl) of the cDNA reaction, with an initial incubation step at 94°C for 1 min. Cycling conditions consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min over a total of 30 cycles. The reaction
was completed by another incubation step at 72°C for 10 min. The PCR products were subjected to electrophoresis in 2% agarose gel and visualised by ethidium bromide.

Evaluation of p73, deltaNp73 and N-myc

N-myc, p73 and deltaNp73 were evaluated by flow cytometry. To allow intracellular staining, tumour cells were fixed and permeabilised by methanol–acetic acid (1:1, −20°C) before the antibodies were added. Monoclonal anti-N-myc antibody was from Calbiochem (clone NCM II 100; mouse IgG1; Calbiochem, Bad Soden, Germany). p73 was measured using monoclonal anti-p73 (clone ER-15, Becton Dickinson). To identify deltaNp73, the monoclonal antibody anti-deltaNp73 (clone 38C674) was purchased from Active Motif (Rixensart, Belgium). Primary antibodies were labelled with goat anti-mouse IgG-FITC. To evaluate background staining, goat anti-mouse IgG-FITC was used.

N-myc was also explored by Western blot analysis using monoclonal antibodies against N-myc (1:250, clone NCM II 100; mouse IgG1). β-Actin (1:1,000, mouse; Sigma, Taufkirchen, Germany) served as the internal control.

To investigate N-myc coding mRNA, RT/PCR has been carried out as described above. The N-myc primer sequences were as follows: forward: 5′GACCAACAAGCCTCAGTAC 3′; reverse: 5′GGGATGGGAAGGCATCGTT 3′.

Statistics

All experiments were performed 3–6 times. Statistical significance was investigated by the Wilcoxon–Mann–Whitney U-test. Differences were considered statistically significant at P<0.05.

RESULTS

VPA downregulates cell adhesion of CDDP- and VCR-resistant UKF-NB-3 tumour cells

Adhesion of UKF-NB-3, UKF-NB-3CDDP, UKF-NB-3VCR or UKF-NB-3DOX was quantified 60 min after plating the cells on to an endothelial cell monolayer (Figure 1). Nearly 200 parental (drug-sensitive) UKF-NB-3 cells mm−2 were attached to HUVEC during this time (SDinterassay<50%, SDintraassay<10%). The amount of adherent cells increased fourfold when UKF-NB-3 became resistant to CDDP or VCR. Doxorubicin resistance did not induce any effects on tumour cell binding to HUVEC. Table 1 provides a profile of cross-resistance among the sublines examined.

The application of 1 mM VPA to UKF-NB-3CDDP or UKF-NB-3VCR significantly blocked the cellular adhesion process (Figure 1). A 5-day incubation period evoked stronger effects than a 3-day incubation period. Notably, treatment of UKF-NB-3CDDP with VPA completely reverted the elevated adhesion behaviour induced by drug resistance. Valproic acid also acted on the parental cell lines, as evidenced by a significant downregulation of the number of adherent UKF-NB-3. The 60-min adhesion rate was reduced by 59.4 ± 18.6% (n=6).

The PicoGreen assay did not reveal any proliferative activity during the experiment, which rules out the possibility that adhesion differences between drug resistant, VPA-treated and control NB cells may be caused by different cell growth capacity.

VPA upregulates NCAM surface expression

Figure 2 depicts one representative histogram analysis of NCAM receptor expression. The histogram presentation concentrates on VPA-treated (5-day treatment)-resistant tumour cells. Results of parental UKF-NB-3 and of resistant tumour cells treated for 3 days with VPA were not inserted, because the histograms overlapped, making the figures unclear. However, the complete experimental data set (MFU ± s.d.; n=6) is given below the histogram.

Table 1 Level of drug resistance, indicated as IC50 values (ng·ml−1)

| Cell line | IC50 VCR | IC50 DOX | IC50 CDDP |
|-----------|----------|----------|-----------|
| UKF-NB-3  | 0.74 ± 0.15 | 8.2 ± 2.5 | 136 ± 41  |
| UKF-NB-3VCR | 47.9 ± 11.2 | 114.6 ± 20.7 | 154 ± 32  |
| UKF-NB-3CDDP | 23.3 ± 6.4 | 40.7 ± 12.3 | 225 ± 38  |
| UKF-NB-3DOX | 1.35 ± 0.47 | 17.4 ± 7.7 | 819 ± 52  |
| UKF-NB-3 | 0.35 ± 0.09 | 2.25 ± 0.78 | 188 ± 22  |
| UKF-NB-3VCR | 52.7 ± 9.8 | 69.1 ± 15.8 | 532 ± 68  |
| UKF-NB-3CDDP | 205 ± 51.2 | 625 ± 15.4 | 231 ± 22  |
| UKF-NB-3DOX | 0.84 ± 0.31 | 17.3 ± 2.4 | 1278 ± 177 |
| UKF-NB-6  | 1.91 ± 0.18 | 3.5 ± 0.9 | 114 ± 32  |
| UKF-NB-6VCR | 57.2 ± 11.2 | 108 ± 12 | 523 ± 69  |
| UKF-NB-6CDDP | 195 ± 3.4 | 17.2 ± 6.5 | 102 ± 18  |
| UKF-NB-6DOX | 3.72 ± 0.76 | 21.4 ± 4.5 | 2102 ± 150 |
| SKNSH      | 7.9 ± 1.3 | 25.4 ± 10.1 | 47.1 ± 13.5 |

Values are from six independent experiments ± s.d.

Tumour cell adhesion

Figure 1 Valproic acid treatment causes adhesion blockade in CDDP- and VCR-resistant NB cells. The figure depicts adhesion capacity of parental UKF-NB-3 (control) vs VCR- (UKF-NB-3VCR), CDDP- (UKF-NB-3CDDP) or DOX-resistant NB subpopulations (UKF-NB-3DOX) vs resistant cell lines treated with 1 mM VPA for 3 (VPA3) or 5 days (VPAS). Neuroblastoma cells were added at a density of 0.5 × 104 cells/well to HUVEC monolayers for 60 min. Non-adherent tumour cells were washed off in each sample, the remaining cells were fixed and counted in five different fields (5 × 0.25 mm2) using a phase contrast microscope. Adhesion capacity is depicted as tumour cell adhesion mm−2 (mean ± s.d.; n=6).
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Valproic acid treatment enhances NCAM surface expression in CDDP- and VCR-resistant NB cells. Histograms plots show NCAM surface expression on untreated vs VPA-treated UKF-NB-3CDDP, UKF-NB-3VCR and UKF-NB-3DOX. IgG isotype controls are also included. The complete experimental data set (MFI ± s.d.; n = 6) is given below the histograms. Tumour cells were disaggregated mechanically in each experiment and washed in blocking solution. An FITC-conjugated monoclonal antibody anti-CD56, clone 16.2, was used to detect the NCAM 120, 140 and 180 kDa isoform. A mouse IgG1-FITC served as the isotype control (IgG). Fluorescence was analysed using a FACScan flow cytometer, and a histogram plot (FL1-Height) was generated to show FITC fluorescence.

**Reduction of NCAM surface expression** was observed on UKF-NB-3CDDP and UKF-NB-3VCR, when compared to the parental control cell line. However, no changes were seen with NCAM expression on UKF-NB-3DOX, compared to the controls. Treatment of CDDP- or VCR-resistant cell lines with VPA led to a significant increase in NCAM, which exceeded the control values after a 5-day incubation period.

Valproic acid also acted on the parental cell lines, as evidenced by a significant upregulation of NCAM by + 56.9 ± 22.8% (n = 5).

**VPA enhances NCAM protein content**

Similar to the modifications of the NCAM surface expression level, UKF-NB-3CDDP and UKF-NB-3VCR were characterised by a strong reduction of NCAM proteins when compared to the parental UKF-NB-3 control cells (Figure 3). No differences were seen between UKF-NB-3 and UKF-NB-3DOX.

When UKF-NB-3CDDP or UKF-NB-3VCR were treated with VPA for 3 or 5 days, NCAM protein content became upregulated, partially exceeding the control values. Applying VPA to UKF-NB-3DOX did not induce any alterations in NCAM, independent of the incubation time.

**VPA modifies NCAM mRNA expression**

Assessment of NCAM mRNA showed distinct expression of mRNA encoding the 140 kDa isoform in UKF-NB-3 control cells, which however became down-modulated in UKF-NB-3CDDP or UKF-NB-3VCR (Figure 4). Only slight differences were visualised between UKF-NB-3 and UKF-NB-3DOX. The presence of VPA was accompanied by elevated 140 kDa mRNA levels in UKF-NB-3CDDP or UKF-NB-3VCR. This effect was not seen in UKF-NB-3DOX, irrespective if VPA was applied for 3 or 5 days.

Messenger ribonucleic acid encoding the 180 kDa isoforms was not detected in parental UKF-NB-3, nor in UKF-NB-3CDDP, or UKF-NB-3DOX, and only very weakly expressed in UKF-NB-3VCR. Surprisingly, VPA evoked NCAM 180 kDa mRNA synthesis already after 3 days in UKF-NB-3DOX. The same phenomenon was observed after a 5-day VPA treatment in UKF-NB-3CDDP or UKF-NB-3VCR.

**VPA alters p73, deltaNp73 and N-myc expression**

p73, deltaNp73 and N-myc have been identified to trigger NCAM expression (Blaheta et al, 2004). They were therefore used as biomarkers to further explore the influence of VPA on NCAM-triggered NB adhesion. p73 was detected in UKF-NB-3, expression of which was significantly reduced in UKF-NB-3CDDP and UKF-NB-3VCR. Doxorubicin resistance was not accompanied by a distinct p73 downregulation (Table 2). When VPA was added to the cell cultures, downregulation of p73 seen in UKF-NB-3CDDP and UKF-NB-3VCR was reverted and the protein became upregulated after 3 and 5 days. deltaNp73 became enhanced in UKF-NB-3CDDP and UKF-NB-3VCR compared to the drug naïve UKF-NB-3 controls. Application of VPA led to a significant reduction of deltaNp73 in CDDP- or VCR-resistant cell lines (Figure 5). Moderate N-myc downregulation was induced by

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**Figure 2** Valproic acid treatment enhances NCAM surface expression in CDDP- and VCR-resistant NB cells. Histograms plots show NCAM surface expression on untreated vs VPA-treated UKF-NB-3CDDP, UKF-NB-3VCR and UKF-NB-3DOX. IgG isotype controls are also included. The complete experimental data set (MFI ± s.d.; n = 6) is given below the histograms. Tumour cells were disaggregated mechanically in each experiment and washed in blocking solution. An FITC-conjugated monoclonal antibody anti-CD56, clone 16.2, was used to detect the NCAM 120, 140 and 180 kDa isoform. A mouse IgG1-FITC served as the isotype control (IgG). Fluorescence was analysed using a FACScan flow cytometer, and a histogram plot (FL1-Height) was generated to show FITC fluorescence.

**Figure 3** Western blot analysis of NCAM from the proteins of UKF-NB-3, UKF-NB-3CDDP UKF-NB-3VCR and UKF-NB-3DOX and resistant subpopulations treated with VPA for 3 (VPA3) or 5 days (VPA5). Cell lysates were subjected to SDS–PAGE and blotted on the membrane incubated with anti-NCAM (clone 16.2) monoclonal antibodies. β-Actin served as the internal control. The figure shows one representative from three separate experiments.

**Figure 4** NCAM protein expression

| Protein | Control | Resistant | VPA3 | VPA5 | Resistant | VPA3 | VPA5 |
|---------|---------|-----------|------|------|-----------|------|------|
| NCAM    |          |           |      |      |           |      |      |
| VPA     |          |           |      |      |           |      |      |
| CDDP    |          |           |      |      |           |      |      |
| VCR     |          |           |      |      |           |      |      |
| DOX     |          |           |      |      |           |      |      |

| Protein | Control | Resistant | VPA3 | VPA5 | Resistant | VPA3 | VPA5 |
|---------|---------|-----------|------|------|-----------|------|------|
| NCAM    |          |           |      |      |           |      |      |
| VPA     |          |           |      |      |           |      |      |
| CDDP    |          |           |      |      |           |      |      |
| VCR     |          |           |      |      |           |      |      |
| DOX     |          |           |      |      |           |      |      |

**Figure 5** VPA alters p73, deltaNp73 and N-myc expression
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Valproic acid (VPA) has long been used to treat neuroblastoma due to its antiproliferative effects. In this study, we investigated the role of VPA in neuroblastoma, focusing on its influence on N-myc expression.

**Figure 4** RT-PCR analysis of NCAM 140 and 180 kDa RNA in UKF-NB-3, UKF-NB-3 CDDP, UKF-NB-3 VCR, UKF-NB-3 DOX and resistant subpopulations treated with VPA for 3 (VPA3) or 5 days (VPA5). Ribonucleic acid were extracted, reverse-transcribed and submitted to semiquantitative RT–PCR analysis. The complete experimental data set (MFU ± s.d.; n = 6) is given below the histogram.

**Table 2** Mean fluorescence units showing influence of VPA on p73 and deltaNp73 expression in drug-resistant neuroblastoma cells

| Cell line | p73 | deltaNp73 |
|-----------|-----|-----------|
| UKF-NB-3  | 18.29 ± 6.25 | 11.51 ± 8.34 |
| CDDP      | 3.38 ± 3.64a | 19.71 ± 7.68a |
| CDDP+VPA3 | 8.16 ± 5.66b | 12.44 ± 6.94b |
| CDDP+VPA5 | 12.8 ± 7.32b | 11.06 ± 6.72b |
| VCR       | 5.23 ± 4.78a | 26.17 ± 13.95a |
| VCR+VPA3  | 13.78 ± 6.13b | 17.33 ± 8.99b |
| VCR+VPA5  | 17.62 ± 8.72b | 11.23 ± 6.85b |
| DOX       | 15.46 ± 8.29  | 10.02 ± 4.29  |
| DOX+VPA3  | 17.90 ± 11.21 | 6.04 ± 6.78   |
| DOX+VPA5  | 19.95 ± 13.96 | 11.95 ± 3.81  |

Values are from six independent experiments ± s.d. CDDP indicates UKF-NB-3 CDDP, VCR indicates UKF-NB-3 VCR, DOX indicates UKF-NB-3 DOX. VPA was added for 3 (VPA3) or 5 days (VPA5). *Indicates significant difference to UKF-NB-3. †Indicates significant difference to the drug-resistant NB sublines.

**Figure 5** Valproic acid alters N-myc expression in CDDP- and VCR-resistant UKF-NB-3 cells. The right part of the figure depicts results from flow cytometry analysis. To allow intracellular staining, tumour cells were fixed and permeabilised by methanol–acetone before antibodies were added. To identify N-myc, the monoclonal anti-N-myc antibody clone NCM II 100 was used. Primary antibodies were labelled with goat anti-mouse IgG-FITC. Background staining was evaluated by goat anti-mouse IgG-FITC. The histograms plots show N-myc expression level in untreated vs VPA-treated UKF-NB-3 CDDP, UKF-NB-3 VCR and UKF-NB-3 DOX. IgG isotype controls are also included. The complete experimental data set (MFU ± s.d.; n = 6) is given alongside. The left part of the figure shows Western blot analysis of N-myc from the proteins of UKF-NB-3, UKF-NB-3 CDDP, UKF-NB-3 VCR, UKF-NB-3 DOX and resistant subpopulations treated with VPA for 3 (VPA3) or 5 days (VPA5). Cell lysates were subjected to SDS–PAGE and blotted on the membrane incubated with anti-N-myc monoclonal antibodies. β-Actin served as the internal control. The figure shows one representative from three separate experiments.
Table 3  Comparative analysis of adhesion capacity, NCAM and N-myc expression level of several neuroblastoma cell lines and drug-resistant sublines

| Cell line | Cell adhesion to HUVEC (cells mm⁻²) | NCAM expression (MFU) | N-myc expression (MFU) |
|-----------|-------------------------------------|-----------------------|------------------------|
| UKF-NB-2  | 75.2                                | 188.2                 | 40.1                   |
| UKF-NB-2+VPA | 60.8³                           | 212.6                 | 28.6³                  |
| UKF-NB-2CDDP | 318.⁴                          | 82.8⁴                 | 39.6                   |
| UKF-NB-2VCR | 175.²                             | 168.9⁴               | 32.²                   |
| UKF-NB-2VCR+VPA | 245.⁶                      | 99.⁷                 | 44.7                   |
| UKF-NB-2CDDP+VPA | 121.⁶                       | 154.⁶                 | 42.3                   |
| UKF-NB-2DOX | 172.⁶                             | 127.⁹                | 38.⁸                   |
| UKF-NB-2DOX+VPA | 124.⁵                      | 143.⁵                | 39.⁴                   |
| UKF-NB-6  | 72.⁴                                | 256.⁸                 | 339.³                  |
| UKF-NB-6+VPA | 72.⁴                               | 256.⁸                 | 339.³                  |
| UKF-NB-6CDDP | 81.⁶                              | 248.⁷                | 305.⁸                  |
| UKF-NB-6VCR | 56.⁸                              | 283.⁸                | 224.²                  |
| UKF-NB-6VCR+VPA | 115.⁶                          | 181.⁰                | 298.⁹                  |
| UKF-NB-6DOX | 79.²⁴                             | 284.²⁴               | 331.⁶                  |
| UKF-NB-6DOX+VPA | 73.⁸                           | 251.³                | 344.⁰                  |
| UKF-NB-6DOX+VPA | 71.⁹                           | 254.⁵                | 321.⁷                  |
| SKNSH     | 56.⁸                              | 318.⁶                | 17.⁷                   |
| SKNSH+VPA | 27.²²                          | 812.²¹               | 18.⁶                   |

MFU = mean fluorescence units. *Indicates significant difference to the parental neuroblastoma cell line. †Indicates significant difference to the drug-resistant tumour sublines. ‡Indicates significant difference between VPA-treated parental cells and their non-treated controls. Mean standard deviations were as follows: cell adhesion intra-assay <25%, cell adhesion inter-assay <80%. NCAM expression intra-assay <5%, NCAM expression inter-assay <30%. N-myc expression intra-assay <5%, N-myc expression inter-assay <50%.

Table 4  Antiproliferative effects of VPA, indicated as IC₅₀ values (mM)

| Cell line | IC₅₀ |
|-----------|------|
| UKF-NB-2  | 0.44±0.31 |
| UKF-NB-2CDDP | 1.58±0.58 |
| UKF-NB-2VCR | 1.36±0.62 |
| UKF-NB-2DOX | 1.44±0.42 |
| UKF-NB-3  | 1.03±0.37 |
| UKF-NB-3CDDP | 1.51±0.44 |
| UKF-NB-3VCR | 1.68±0.57 |
| UKF-NB-3DOX | 2.18±0.76 |
| UKF-NB-6  | 1.27±0.53 |
| UKF-NB-6CDDP | 1.53±0.46 |
| UKF-NB-6VCR | 2.36±0.71 |
| UKF-NB-6DOX | 0.93±0.39 |
| SKNSH     | 3.16±0.78 |

Values are from six independent experiments ± s.d.

In fact, several cases have been documented showing antineoplastic effects of VPA in patients with relapsed tumours. When VPA was given as maintenance therapy for childhood malignant glioma after postoperative combined chemotherapy and irradiation, about 10% of these patients were maintained in continuous complete remission and an equal number of patients showed at least partial responses (Blaheta et al., 2005). An additional pediatric patient with glioblastoma multiforme responded to VPA after showing progressive disease shortly after having received combined chemotherapy and irradiation as well as topotecan (Witt et al., 2004). Another pediatric patient suffering from a relapsed supratentorial primitive neuroectodermal tumour while receiving chemotherapy (CCNU, VCR and cisplatinum) after total resection and irradiation showed conspicuous signs of glial differentiation induction and a non-malignant morphology on histological examination. This patient had received VPA for epilepsy treatment for a period of several months before the tumour recurred (Driever et al., 2004).

In vitro, VPA has been shown to inhibit proliferation in acute myeloid leukaemia cells expressing P-glycoprotein (P-gp) and MDR-associated protein 1 (Tang et al., 2004), and to increase sensitivity towards apoptosis in hepatoma cells resistant to etoposide (Schuchmann et al., 2006). Although the underlying mode of action has not been explored in these studies, the data clearly indicate that VPA may alter the malignant behaviour of tumours that do not respond to chemotherapy. With particular emphasis on NB, VPA significantly prevented the interaction between tumour cells and endothelium. This finding is important, because binding of single cancer cells to the vessel wall represents the first step in the haematogenous invasion cascade proceeding transendothelial migration and invasion into surrounding tissue. We therefore conclude that VPA may have a direct impact on metastasis formation. In good accordance to this hypothesis, VPA enhanced the NCAM surface level, expression of which is strongly involved in tumour cell adhesion and penetration.

In primitive neuroectodermal tumour cells, an increase in NCAM was paralleled by a significant reduction in cellular motility and adhesion capacity (Owens et al., 1998; Prag et al., 2002). In a rat model, NCAM-transfected glioma tumour cells became less invasive and destructive than control cells with a low NCAM expression level (Edvardsen et al., 1994). Diminished expression of NCAM was also associated with clinically aggressive colon cancers (Sampson-Johannes et al., 1996; Roessler et al., 1997; Huerta et al., 2001), and dissemination of pancreatic β-tumour cells (Perl et al., 1999; Cavallaro et al., 2001). Tezel et al. (2001) suggested that NCAM expression in tubular adenocarcinoma of the pancreas has a significant impact on overall patient survival. We recently demonstrated an inverse correlation between NCAM expression and NB cell adhesion, assessed on 11 NB cell lines. In particular, transfection with a cDNA encoding the human NCAM-140KD
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To summarise, evidence is presented showing that drug-resistant NB cells are sensitive to VPA treatment. Valproic acid distinctly reduced the invasive properties of NB and may therefore be well suited to amend the current treatment protocol with particular emphasis on those tumours that do not respond to chemotherapy. Nevertheless, our data are particularly limited to four NB cell lines. Therefore, the hypothetical possibility that VPA might overcome drug resistance in general needs further investigation. Remarkably, VPA blocked cell adhesion of parental, but not of DOX-resistant UKF-NB-3 or UKF-NB-6 tumour cells in our assay. This implies that DOX may, under certain circumstances, induce resistance to VPA. A similar phenomenon was found using NB cells with ‘naturally’ arrested DOX resistance, SMS-KANR and SMS-KCNR (Reynolds et al. 1986). Tabe et al (2006) demonstrated in this context that the HDAC-inhibitor FK228 induces P-gp expression and prevents growth inhibition and apoptosis in acute promyelocytic leukaemia cells subsequently incubated with DOX. Okada et al (2006) observed resistance development in DOX-resistant clones of osteosarcoma and Ewing’s family of tumours after exposure to FK228. Nevertheless, FK228 is chemically different from VPA and, therefore, further experiments are necessary to explore this issue.

Differences between VCR/CDDP- and DOX-resistant cell lines may also point to different resistance mechanisms that are operational in these cell lines. Suppression of MAP kinase (MEK-ERK) signalling has been observed in NB cells with acquired resistance to DOX (Mattingly et al. 2001; Armstrong et al. 2006). Down-modulation of ERK1/2 phosphorylation has further been documented in NB cells with acquired resistance to CDDP or VCR. However, these cell lines were additionally characterised by a distinct Akt activation (Kotchetkov et al., 2005; Servidei et al., 2006). Therefore, although purely speculative, fine-tuned alterations of the ERK and Akt signalling system may be – at least partially – responsible for establishing VCR and CDDP, but not DOX resistance in our NB cell model. However, detailed knowledge of resistance mechanism in individual cancer cells is necessary to allow better prediction of the clinical use of VPA.

Other HDAC inhibitors have also been shown to inhibit tumour growth and to overcome multidrug resistance. Notably, suberoylanilide hydroxamic acid (SAHA) has been demonstrated to act on VCR-resistant leukaemia cell lines (Rueff et al., 2002), adriamycin-resistant breast (Castro-Galache et al., 2003) and paclitaxel-resistant ovarian cancer cells (Sonnemann et al., 2006). SAHA at 5 μM significantly diminished UKF-NB-3(CDDP) or UKF-NB-3(VCR) proliferation and cell adhesion to HUVEC in our own experiments (data not shown). Histone deacetylase inhibitors different from VPA may therefore be considered to become additional options for the treatment of drug-resistant NB. However, detailed studies are necessary to explore their clinical value.

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REFERENCES

Akeson R, Bernards R (1990) N-myc down regulates neural cell adhesion molecule expression in rat neuroblastoma. Mol Cell Biol 10: 2012 – 2016
Armstrong MB, Bian X, Liu Y, Subramanian C, Ratanaproeksa AB, Shao F, Yu VC, Kwok RP, Opipari Jr AW, Castle VP (2006) Signalling from p53 to NF-kappaB determines the chemotherapy responsiveness of neuroblastosoma. Neoplasia 8: 967 – 977
Blaheta RA, Kronenberger B, Woitaschek D, Weber S, Scholz M, Schuldes H, Encke A, Markus BF (1998) Development of an ultrasensitive in vitro
Translational Therapeutics

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Okada T, Tanaka K, Nakatani F, Sakimura R, Matsunobu T, Li X, Hanada M, Nakamura T, Oda Y, Tsuneyoshi M, Iwamoto Y (2006) Involvement of P-glycoprotein and MRPI in resistance to cyclic tetrapeptide subfamily of histone deacetylase inhibitors in the drug-resistant osteosarcoma and Ewing’s sarcoma cells. Int J Cancer 118: 90 – 97

ancias GC, Orr EA, DeMasters BK, Muschel RJ, Berens ME, Kruse CA (1998) Overexpression of a transmembrane isoform of neural cell adhesion molecule alters the invasiveness of rat CNS-1 glioma. Cancer Res 58: 2020 – 2028

Pathil A, Armeanu S, Venturelli S, Mascalzi P, Weiss TS, Gregor M, Lauer UM, Bitzer M (2006) HDAC inhibitor treatment of hepatoma cells induces both TRAIL-independent apoptosis and restoration of sensitivity to TRAIL. Hepatology 43: 425 – 434

Per AL, Dahl U, Wilgenbus P, Cremer H, Semb H, Christofori G (1999) Reduced expression of neural cell adhesion molecule induces metastatic dissemination of pancreatic beta tumor cells. Nat Med 5: 286 – 291

Prag S, Lepekhin EA, Kolkova K, Hartmann-Petersen K, Awa W, Malmod PS, Belman V, Gallagher HC, Berezin V, Bock E, Pedersen N (2002) NCAM regulates cell motility. J Cell Sci 115: 283 – 292

Reynolds CP, Biedler JL, Spengler BA, Reynolds DA, Ross RA, Frenkel EP, Smith RG (1986) Characterization of human neuroblastoma cell lines established before and after therapy. J Natl Cancer Inst 76: 375 – 387

Roesler J, Srivatsan E, Moatamed F, Peters J, Livingston EH (1997) Tumor suppressor activity of neural cell adhesion molecule in colon carcinoma. Am J Surg Pathol 21: 251 – 257

Ruell A, Bernhard D, Tainton KM, Kohler F, Smyth MJ, Johnstone RW (2002) Suberoylanilide hydroxamic acid (SAHA) overcomes multidrug resistance and induces cell death in P-glycoprotein-expressing cells. Int J Cancer 99: 292 – 298

Sampson-Johnson A, Wang W, Shitvalman E (1996) Colonization of human lung grafts in SCID-hu mice by human colon carcinoma cells. Int J Cancer 65: 864 – 869

Schuchmann M, Schulze-Bergkamen H, Fleischer B, Schattgen JM, Siebler J, Weinmann A, Fiebig A, Lohse AW, Galle PR (2006) Histone deacetylase inhibition by valproic acid down-regulates c-FLIP/CASH and sensitizes hepatoma cells towards CD95- and TRAIL-receptor-mediated apoptosis and chemotherapy. Oncol Rep 15: 227 – 230

Servidei T, Riccardi A, Sanguinetti M, Dominici C, Riccardi R (2006) Increased sensitivity to the platelet-derived growth factor (PDGF) receptor inhibitor STI571 in chemoresistant glioma cells is associated with enhanced PDGF-BB-mediated signaling and STI571-induced Akt inactivation. J Cell Physiol 208: 220 – 228

Sonnenmann J, Gange I, Pilz S, Stotzer C, Oehlering R, Belau A, Lorenz G, Beck JF (2006) Comparative evaluation of the treatment efficacy of suberoylanilide hydroxamic acid (SAHA) and paclitaxel in ovarian cancer cell lines and primary ovarian cancer cells from patients. BMC Cancer 6: 1246 – 1251

Tabe Y, Konopleva M, Contractor R, Munsell M, Schober WD, Jin L, Tsutsumi-Ishii Y, Nagaoa I, Iagi J, Andreff M (2006) Up-regulation of MDR1 and induction of doxorubicin resistance by histone deacetylase inhibitor depsipeptide (FK228) and ATRA in acute promyelocytic leukemia cells. Blood 107: 1546 – 1554

Tang R, Faussett AM, Majdak P, Perrot JY, Chaudhuri D, Legrand O, Marie JP (2004) Valproic acid inhibits proliferation and induces apoptosis in acute myeloid leukemia cells expressing P-gp and MRPI. Leukemia 18: 4315 – 4321

Tezel E, Kawase Y, Takeda S, Oshima K, Nakao A (2001) Expression of neural cell adhesion molecule in pancreatic cancer. Pancreas 22: 122 – 125

Tsurutani J, Soda H, Oka M, Suenaga M, Doi S, Nakamura Y, Nakatomi K, Shiozawa K, Amada YY, Kamihira S, Kohno S (2003) Antiproliferative effects of the histone deacetylase inhibitor FR901228 on small-cell lung cancer lines and drug-resistant sublines. Int J Cancer 104: 238 – 242

Witt O, Schweigerer L, Driever PH, Wolff J, Pekrun A (2004) Valproic acid treatment of glioblastoma multiforme in a child. Pediatr Blood Cancer 43: 181

assay to monitor growth of primary cell cultures with reduced mitotic activity. J Immunol Meth 211: 159 – 169

Blaheta RA, Hunderm E, Mayer G, Vogel JU, Kornhuber B, Cinatl J, Markus BH, Driever PH, Cinatl Jr J (2002) Expression level of neural cell adhesion molecule (NCAM) inversely correlates with the ability of neuroblastoma cells to adhere to endothelium in vitro. Cell Commun Adhes 9: 131 – 147

Blaheta RA, Beecken WD, Engl T, Jonas D, Oppermann E, Hunderm E, Doerr HW, Scholz M, Cinatl J (2004) Human cytomegalovirus infection of tumor cells downregulates NCAM (CD56): a novel mechanism for virus-induced tumor invasiveness. Neoplasia 6: 323 – 331

Blaheta RA, Michaelis M, Driever PH, Cinatl Jr J (2005) Evolving anticancer drug valproic acid: insights into the mechanism and clinical studies. Med Res Rev 25: 383 – 397

Castro-Galache MD, Ferragut JA, Barbera VM, Martin-Orozco E, Gonzalez-Ros JM, Garcia-Morales P, Saceda M (2003) Susceptibility of multidrug resistance tumor cells to apoptosis induction by histone deacetylase inhibitors. Int J Cancer 104: 579 – 586

Cavallaro U, Niedermeyer J, Fuixa M, Christofori G (2001) N-CAM modulates tumour-cell adhesion to matrix by inducing FGFR-receptor signalling. Nat Cell Biol 3: 650 – 657

Cinatl Jr J, Cinatl J, Scholz M, Driever PH, Henrich D, Kabickova H, Vogel JU, Doerr HW, Kornhuber B (1996) Antitumor activity of sodium valproate in cultures of human neuroblastoma cells. Anticancer Drugs 7: 766 – 773

De Laurenci V, Raschella G, Barcaroli D, Annichiarico-Petruszelli M, Ranalli M, Catani MV, Tanno B, Costanzo A, Levrero M, Melino G (2000) Induction of neuronal differentiation by p73 in a neuroblastoma cell line. J Biol Chem 275: 15226 – 15231

Driever PH, Wagner S, Hofstadter F, Wolff JE (2004) Valproic acid induces differentiation of a supratentorial primitive neuroectodermal tumor. Pediatr Hematol Oncol 21: 743 – 751

Edwardsen KD, Pedersen P-H, Bjerkvig R, Hermann GG, Zeuthen J, Laerum AK, Driever P, Klingebiel T, Cinatl Jr J (2003) Development of resistance to histone deacetylase inhibitors LBH589 is a potent antimyeloma agent that overcomes drug resistance in neuroblastoma cells with acquired multi-drug resistance does not depend on P-gp expression. Int J Oncol 27: 1029 – 1037

Maison P, Carvajal-Vergara X, Ocio EM, Lopez-Perez R, Mateo G, Gutierrez N, Atadja P, Pandelila A, San Miguel JF (2006) The histone deacetylase inhibitor LBH589 is a potent antiyminema agent that overcomes drug resistance. Cancer Res 66: 5781 – 5789

Mattingly RR, Milstein ML, Mirkin BL (2001) Down-regulation of growth factor-stimulated MAP kinase signaling in cytotoxic drug-resistant human neuroblastoma cells. Cell Signal 13: 499 – 505

Kotchetkov R, Driever PH, Cinatl J, Michaelis M, Karakasova J, Blaheta R, Souire J-A, Von Deimling A, Moog J, Cinatl Jr J (2005) Increased malignant behavior in neuroblastoma cells with acquired multi-drug resistance does not depend on P-gp expression. Int J Oncol 27: 1029 – 1037

Tsutsumi-Ishii Y, Nagaoka I, Igari J, Andreeff M (2006) Up-regulation of MDR1 and induction of doxorubicin resistance by histone deacetylase inhibitor depsipeptide (FK228) and ATRA in acute promyelocytic leukemia cells. Blood 107: 1546 – 1554

Tang R, Faussett AM, Majdak P, Perrot JY, Chaudhuri D, Legrand O, Marie JP (2004) Valproic acid inhibits proliferation and induces apoptosis in acute myeloid leukemia cells expressing P-gp and MRPI. Leukemia 18: 4315 – 4321

Tezel E, Kawase Y, Takeda S, Oshima K, Nakao A (2001) Expression of neural cell adhesion molecule in pancreatic cancer. Pancreas 22: 122 – 125

Tsurutani J, Soda H, Oka M, Suenaga M, Doi S, Nakamura Y, Nakatomi K, Shiozawa K, Amada YY, Kamihira S, Kohno S (2003) Antiproliferative effects of the histone deacetylase inhibitor FR901228 on small-cell lung cancer lines and drug-resistant sublines. Int J Cancer 104: 238 – 242

Witt O, Schweigerer L, Driever PH, Wolff J, Pekrun A (2004) Valproic acid treatment of glioblastoma multiforme in a child. Pediatr Blood Cancer 43: 181

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