# Valproic acid induces non-apoptotic cell death mechanisms in multiple myeloma cell lines

CHANTAL SCHWARTZ[1], VALERIE PALISSOT[1], NASSERA AOUALI[1], SEVERINE WACK[1], N.H.C. BRONS[1], BERNADETTE LENERS[1], MANON BOSSELER[1] and GUY BERCHEM[2]

[1]Laboratoire d'Hémato-Cancérologie Expérimentale, CRP-Santé, 84 Val Fleuri, L-1526 Luxembourg; [2]Service d'Hémato-Cancérologie, Centre Hospitalier Luxembourg, 4 rue Barblé, L-1210 Luxembourg

Received October 13, 2006; Accepted November 27, 2006

**Abstract.** Multiple myeloma (MM) is an incurable hematological disorder characterized by dysregulated proliferation of terminally differentiated plasma cells. Aberrant histone acetylation has been observed in the development of numerous malignancies. Histone deacetylase inhibitors such as valproic acid (VPA) are promising drugs for cancer therapy since they have been reported to have antiproliferative effects and to induce differentiation in carcinoma and leukemic cells. Considering the advantage of being already in clinical use for epilepsy treatment, valproic acid might be a promising therapeutic candidate drug in the management of multiple myeloma. In this study, we show that the short fatty acid VPA has a time and dose-dependent cytotoxic effect on the MM cell lines OPM2, RPMI and U266. The influence of VPA on cell cycle and apoptosis have been evaluated by flow cytometry. Our results show that the three cell lines are blocked in $G_0/G_1$ phase. The observed sensitivity to VPA can be partially explained by late apoptosis. Since caspase 3 is activated in all tested cell lines after VPA treatment, a caspase-dependent pathway seems to be involved but not activated by the classic apoptotic pathways. We have also studied another mechanism of cell death, the senescence-like phenotype, but did not find any evidence for its implication. Thus, treatment with VPA may imply other alternative cell death mechanisms.

## Introduction

Multiple myeloma (MM) is, despite the emergence of new treatments in recent years, still a non-curable disease in 2006. It is characterized by the presence of malignant plasma cells predominantly located in the bone marrow. Existing treatments mainly attempt to reduce the malignant cell masses and to overcome the disease-related complications. Whereas initial chemotherapeutic treatment can be successful, drug resistance often develops during disease progression, requiring the use of different drugs. Over the last several years, significant insight into the dysregulation of various signal transduction pathways of MM has led to the development of new agents.

Two new drug classes have radically changed the management of multiple myeloma. Thalidomide analogues and bortezomib are in the process of overthrowing the current paradigm of anthracycline, vinca alkaloid and dexamethasone-based chemotherapy followed by autologous bone marrow transplantation (1).

Cell proliferation and cell death induction are two major targets in cancer therapy. There are two fundamental types of cell death, apoptosis and necrosis, which can be defined by morphological and biochemical criteria. Over the past several years the idea that cells can commit suicide by mechanisms other than apoptosis has been gaining momentum (2-4). Apoptosis is marked by cellular shrinking, condensation of the chromatin and loss of plasma membrane integrity resulting in breaking up of the cell into apoptotic bodies. Whereas apoptosis is an inherent, programmed cellular death mechanism, its conceptual counterpart, necrosis, is a more uncontrolled form of death and is characterized by cellular swelling and disruption of the plasma membrane, leading to release of the cellular components and inflammatory tissue response (5). Alternative models of programmed cell death (PCD) have therefore been proposed, including autophagy, senescence and mitotic catastrophe. Autophagy is the major cellular route for degrading long-lived proteins and cytoplasmic organelles, and the catabolic advantage of increased autophagy might be critical in various stress conditions (6). Briefly, a double membrane vesicle is formed in the cytosol that encapsulates whole organelles and bulk cytoplasm. These autophagosomes then fuse with lysosomes where the content is degraded and recycled (6). The distinction between these different types of death is not always clear. A recent report suggests that autophagy and apoptosis seem to be interconnected by 'molecular switches'. Cellular senescence has been identified as one of the mechanisms mediating the anticancer effects of chemotherapies (7). Morphologic changes observed during this phenomenon are flattening of cells with increase in granularity and biochemical activity of ß-galactosidase. Roninson *et al* (8) defined mitotic catastrophe as a type of cell death resulting

*Correspondence to:* Dr Guy Berchem, Service d'Hémato-Cancérologie, Centre Hospitalier Luxembourg, 4 rue Barblé, L-1210 Luxembourg
E-mail: berchem.guy@chl.lu

*Key words:* multiple myeloma, valproic acid, cell death

from abnormal mitosis usually characterized by formation of large cells with multiple micronuclei and decondensed chromatin.

Histone deacetylase inhibitors represent a novel class of therapeutic agents that regulate genes through specific enzymes. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) determine the acetylation state of the histones, the conformational state of chromatin and, consequently, gene expression (9). This results in histone hyperacetylation, facilitating the access of the transcription factors and leading to an increase in gene expression. Aberrant histone acetylation has been observed in the development of numerous malignancies. Thus, HDAC inhibitors are emerging as promising new treatment strategies in hematologic and other malignancies (10,11).

Valproic acid (VPA) is clinically used in the treatment of epilepsy. In 1997, VPA emerged as an antineoplastic agent, when findings indicated that VPA was able to inhibit proliferation and induced differentiation of neuroectodermal tumor cells *in vivo* (12). Hence, further study demonstated that the antineoplastic effects of VPA are mediated through inhibition of class I HDAC (13) and that its inhibitory function is combined with moderate side-effects (14). VPA has been described as an inducer of apoptosis and differentiation in various cancer types and leukemia (15-18). In a study performed on several leukemic and B cell precursor cell lines, VPA induced apoptosis mediated by caspase-dependent and -independent pathways (19).

In the present study, we investigated the influence of VPA on IL-6 independent multiple myeloma cell lines to gain more insight into the cell death mechanisms triggered by VPA.

## Materials and methods

*Chemicals*. Valproic acid (Depakine®, Aventis) was dissolved at 2 M in sodium chloride solution as a stock solution and diluted to the required concentration with complete medium. Z-VAD-FMK and $C_2$-ceramide were obtained from Alexis Biochemicals, (Illkirch, France) and were dissolved in dimethylsulfoxide. Hoescht 33342 and monodansylcadaverine were from Sigma and respectively dissolved in PBS and acetic acid. Stock preparations of reagents were stored at -20˚C.

*Cell lines*. The human multiple myeloma cell lines OPM2, RPMI-8226 and U266 were obtained from the DSMZ, Braunschweig, Germany. Flow cytometry was used to detect CD marker expression in the three cell lines. The samples were measured on a FACSCanto (Becton Dickinson) and up to 6 markers per tube were analysed by the DivaSoftware (Becton Dickinson). Calibration curves for the mean fluorescence intensities (MFI) were obtained with Fluorospheres (DakoCytomation, K0110). More than 20 CD markers have been used to characterize multiple the respective myeloma cell lines. Mean values (of MFI) of the most discriminant are presented in Table I.

*Cell culture*. Cell lines OPM2, RPMI and U266 were resuspended at a density of $3x10^5$ cells/ml in RPMI-1640 medium (BioWhittaker) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine at 2 mM, 100 U/ml Penicillin G, and 100 mg/ml Streptomycin (Cambrex). All cultures were maintained under a fully humidified atmosphere of 95% air-5% $CO_2$ at 37˚C. Exponentially growing cells were used in all experiments. Cell viability was assessed by the ability to exclude trypan-blue (0.5% w/v, Sigma). Cells were seeded at $3x10^5$ cells/ml in medium with valproic acid at various concentrations.

Table I. Immunophenotyping of the different cell lines.

| | CD19 | CD28 | CD38 | CD45 | CD138 |
|---|---|---|---|---|---|
| OPM2 | 91 | 1158 | 1745 | 169 | 6958 |
| RPMI | 149 | 3636 | 18509 | 881 | 1351 |
| U266 | 136 | 8327 | 254 | 3312 | 2821 |

*Cell viability (XTT) assay*. Briefly, 100 $\mu$l of cell suspension ($1x10^5$ cells/ml) was added to a 96-well plate. Cells were maintained at 37˚C in absence of the drug for 1 day. Various concentrations of VPA were added and cells were incubated for 24 and 48 h. Cell viability was determined by addition of 50 $\mu$l of 1 mg/ml XTT (Sigma) and further incubation for 4 h at 37˚C. Finally, the absorbance of the dye was measured spectrophotometrically at 450 and 630 nm as a reference wavelength.

For investigation of the apoptosis dependence on caspases, the cells were simultaneously incubated with different concentrations of VPA and 100 $\mu$M ZVAD-FMK and the assay was performed as described above.

*VPA stability assay*. VPA stability assay was performed using the COBAS® INTEGRA drug monitoring kit (Roche Diagnostics) according to the manufacturer's instructions. This method uses fluorescence polarization, which is a reproducible function of the drug concentration and is suitable for the quantitative determination of drug concentration for the purpose of therapeutic drug monitoring.

*Propidium-iodide (PI) staining for cell cycle analysis*. PI is a fluorescent nucleic acid binding dye that binds preferentially to double-stranded nucleic acids, allowing fluorescence intensity to be used as an indicator of the cellular DNA content (21,22). After treatment with or without VPA, cells were fixed in 70% ethanol for 30 min at 4˚C. Cells were washed with PBS, pretreated with 1 U RNase, DNase-free, for 30 min at 37˚C, chilled on ice to 4˚C, and stained with 1 $\mu$g/ml PI in PBS. PI stained cells were analyzed using a Becton Dickinson FACSCanto flow cytometer.

*Protein immunoblotting*. VPA-treated and control cells were lysed on ice with a HEPES based lysis buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 1% v/v Nonidet, 0.25% m/v sodium deoxycholate, 1 mM EGTA, 1 mM EDTA pH 8.0, 1 mM PMSF, Complete Protease inhibitor from Roche) for 15 min and protein concentrations were determined by a Bradford assay (Bio-Rad). Protein samples (25 $\mu$g/lane) were separated on 14% SDS-polyacrylamide gels, then transferred onto a nitro-

cellulose membrane (Amersham). Membranes were incubated overnight at 4˚C with caspase 9 (Alexis;1/2000), caspase 3 (1:500) rabbit polyclonal antibodies (Dako), caspase 8 (Alexis; 1/500) mouse monoclonal antibody or actin mouse monoclonal antibodies AC-15 (Sigma; 1/50000). Proteins were visualized using goat anti-rabbit secondary antibodies (Jackson; 1/100000 and 1/60000 respectively) or goat anti-mouse secondary antibodies (Jackson; 1/250000) and detected with the ECL advance detection kit (Amersham).

*DNA fragmentation assay*. After appropriate exposure, cells were collected at densities of $5x10^5$ cells/sample, lysed in 30 $\mu$l of buffer (150 mM NaCl, 10 mM Tris-HCl, 10 mM EDTA, pH 8.0, 0.5 mg/ml proteinase K, 0.5% SDS) and incubated at 50˚C for 2 h. RNase was added at a concentration of 0.1 mg/ml and samples were incubated at 37˚C for 1 h. Samples were loaded onto a 2% agarose gel and migration was performed at 24 V overnight. A methodology positive control was obtained from Jurkat cells treated with 50 $\mu$M VP16 for 6 h (data not shown).

*Flow cytometry analysis for apoptosis quantification*. Annexin V-APC is a sensitive probe for identifying apoptotic cells (23). From VPA-treated and untreated cell cultures, suspensions were made ($1x10^6$ cells/ml) in 0.2 $\mu$m filtered 1X Annexin binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Cell samples of 100 $\mu$l were stained with 5 $\mu$l Annexin V-APC (BD Bioscience) and 1 $\mu$g/ml propidium-iodide (PI, Sigma), incubated for 15 min in the dark and measured with a Becton Dickinson (BD) FACSCanto flow cytometer. The degree of annexin V and PI staining was evaluated on lymphocyte gated cells.

*SA-ß-gal activity*. Cells were stained for ß-galactosidase activity as described by Dimri *et al* (21). Briefly cells were seeded out in 6-wells at densities of $3x10^5$/ml. After appropriate exposure, cells were fixed on poly-L-lysine coated coverslips with 0.5% glutaraldehyde in PBS for 5 min. Cells were stained for 12 h at 37˚C in X-gal staining solution (1 mg/ml X-gal, 40 mM citric acid/sodium phosphate pH 6.0, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, 2 mM $MgCl_2$). As a methodology positive control for ß-galactosidase expression, MCF-7 cells were exposed for 2 h to 1 $\mu$M adriamycin and expression of ß-galactosidase was assessed 4 days after adriamycin treatment (data not shown). Cells were examined by visual light microscopy and the percentage of positively stained cells was determined after counting two random fields of 100 cells each.

*Fluorescent staining*. Different cell lines were seeded out in 12 well plates at $1x10^5$ cells/ml and cultivated for 24 h. Cells were treated with 1 mM of VPA or 2 $\mu$M TSA for 48 h. The plates were centrifuged and the medium was removed.

*Apoptotic body detection*. Cells were stained with 1 mM of Hoescht 33342 for 1 h at 37˚C and analyzed by fluorescence microscopy (DMI 6000 B, Leica).

*MonoDansylCadaverine (MDC) staining of autophagic vacuoles*. Cells were placed onto polylysine coated Labtek chambers, labeled with 100 $\mu$M MDC in PBS for 1 h at 37˚C and washed 3 times with PBS. Analysis of the stained cells was performed by confocal microscopy (LSM 510 Meta, Zeiss).

## Results

*Valproic acid has variable cytotoxic effects on multiple myeloma cell lines*. The cytotoxic effect of valproic acid (VPA) was tested on the multiple myeloma (MM) cell lines OPM2, RPMI, and U266 using an XTT-based cytotoxicity assay. As depicted in Fig. 1A, VPA showed dose- and time-dependent cytotoxicity on all three MM cell lines. OPM2 was the most sensitive cell line to VPA with an $IC_{50}$ of 1 mM VPA after a 48-h treatment, whereas RPMI cells had a respective $IC_{50}$ of 1.85 mM. The U266 cell line was less sensitive to VPA showing a respective $IC_{50}$ of 9.4 mM.

We assessed the stability of VPA in the cell cultures. VPA was added at 0.2 mM to the cell culture or to the medium without cells and incubated over a time period of 5 days in the incubator. The concentrations of VPA were measured from the supernatant of the cell cultures and the VPA containing medium by using the COBAS INTEGRA drug monitoring system from Roche. As depicted in Fig. 1B, no significant modulation of VPA concentration was detectable after 5 days of incubation at 37˚C in all samples. Controls showed an autoabsorbance corresponding to a VPA concentration of 0.03 mM.

*VPA induces a cell cycle arrest in $G_0/G_1$ phase*. In order to verify if VPA has effects on the cell cycle, permeabilized cells of all three cells lines were stained with PI after VPA treatment and their DNA content was analyzed by flow cytometry. Measurements were performed on controls and on cells treated with 1 mM VPA for 48 h. As shown in Fig. 2 (upper), VPA clearly diminishes the cycling cell population undergoing $G_2$/M or S phase in all three cell lines. Here, similar values were obtained for VPA-treated OPM2 and U266 with a respective diminution of cycling cells of 25% and 22% when compared to the controls. In contrast, the difference between control and treated RPMI is not so pronounced showing a cell cycling reduction of 10% only. Our results demonstrate that VPA causes a cell cycle arrest in $G_0/G_1$ phase in OPM2, RPMI and U266 cells.

*Caspase 3 but not caspases 9 and 8 are activated upon VPA treatment, and the pan-caspase inhibitor ZVAD-FMK does not block VPA-induced cell death*. In the next experiment, we addressed the question of whether the cellular death mechanism induced by VPA on the MM cell lines could be caspase-dependent. Therefore we performed immunoblotting with antibodies against caspases 9, 8 and 3 on total protein extracts obtained from lysates of OPM2, RPMI and U266 cell lines treated with 1 mM VPA during 72 h. Controls were cultured in equal conditions but without VPA. As depicted in Fig. 3A, only procaspase 3 was processed to its active form in all three cell lines. In OPM2 caspase 3 cleavage was slightly detected after 24 h, its signal increased at later time-points. In RPMI and U266 the cleaved form of caspase 3 had appeared already at 24 h post VPA treatment. However, no processing

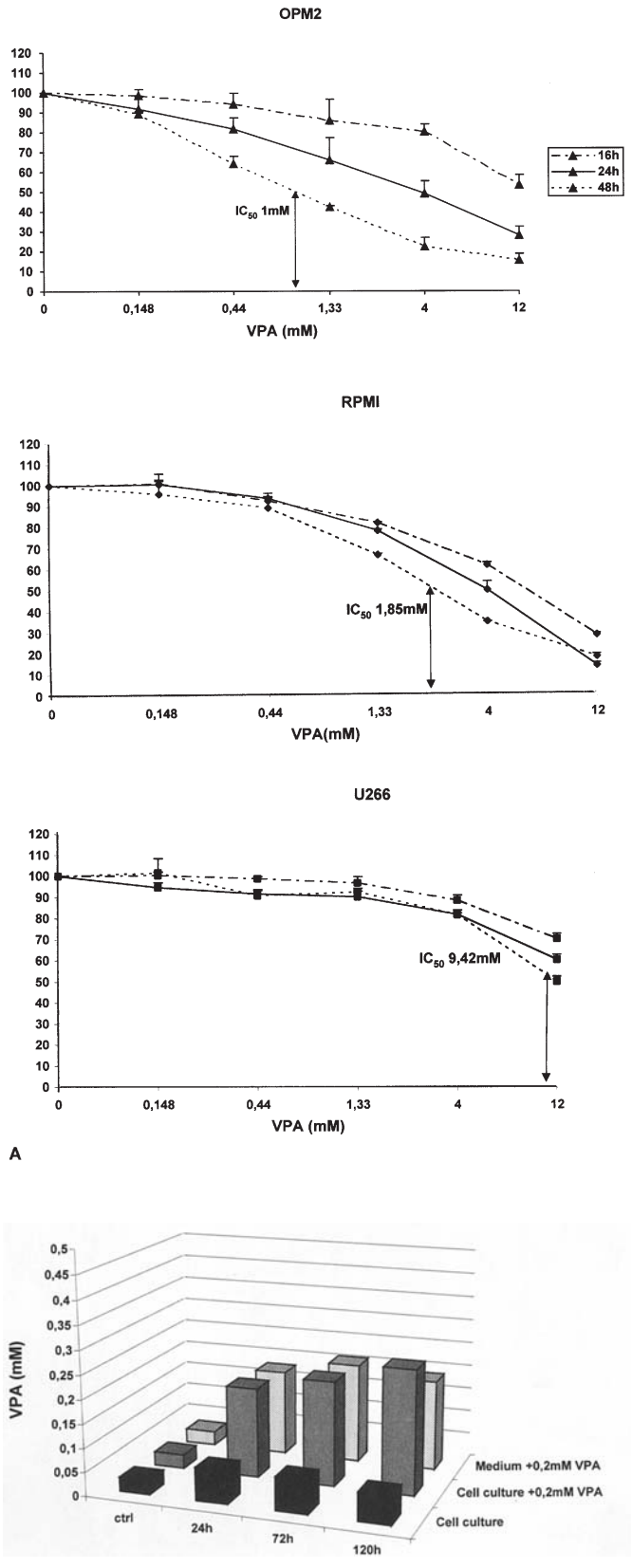



Figure 1. Cell viability assay. (A) The multiple myeloma cell lines OPM2, RPMI 8226 and U266 were treated for varying times and with varying VPA concentrations. Cell viability was assessed by the XTT assay. Results are presented by percentage of viable untreated control. Values represent the means ± SD from three independent experiments. (B) Stability of VPA was tested by addition of 0.2 mM doses to cultured cells and medium alone. Cell culture, supernatant of cultured cells without VPA; cell culture + 0.2 mM VPA, supernatant of cultured cells with 0.2 mM VPA; medium + 0.2 mM VPA, culture medium without cells but with 0.2 mM VPA treated under same conditions as previous samples.

of caspases 9 and 8 were observed in any of the tested cell lines.

In order to verify if cell death upon VPA exposure is caspase-dependent, we performed the incubations in presence of ZVAD-FMK, a pan-caspase inhibitor. Consequently, the most sensitive cell lines OPM2 and RPMI were treated with increasing doses of VPA in presence of ZVAD-FMK within a time-course of 48 h. As depicted in Fig. 3B ZVAD inhibited, but did not completely abolish cell death induced by VPA. Strikingly, ZVAD-FMK seems to have no significant protective effect on OPM2. Calculated $IC_{50}$ values for RPMI were 2.9 and 1.5 mM of VPA with or without ZVAD-FMK respectively, showing a protective effect of ~50%. In conclusion these data indicate that VPA-induced cell death in the tested MM cell lines is not only dependent on caspase activation because ZVAD-FMK was not able to block cell death.

*VPA treatment induces an accumulation of annexin V/PI-positive cells*. Annexin V is a commonly used marker to detect cells undergoing apoptosis in an early stage which preceeds the loss of membrane integrity. It is typically used in combination with a vital dye such as propidium-iodide (PI), allowing to distinguish annexin V-single positive cells undergoing early apoptosis from those subjected to necrotic processes, thus annexin V/PI-double positive cells. Prior to analysis by flow cytometry, cells were labelled with annexin V and propidium-iodide after the indicated incubation time-points with 1 mM VPA. As depicted in Fig. 4A, no significant differences were observed by comparing the amounts of annexin V-single positive VPA-treated cells to the controls. Instead of shifting first to the upper left square, where the annexin V-single positive cells are gated, cells immediately shifted towards the upper right, annexin V/PI-double positive gate. Moreover this accumulation of annexin V/PI-double positive cells was observed for all three cell lines even at early time-points (Fig. 4B), indicating that the appearance of the double positives is not only a consequence of late apoptotic events. Even measurements at 6 h post VPA treatment did not increase significantly the amounts of annexin V-single positive cells, showing instead similar levels between controls and VPA-treated samples (data not shown). Moreover, DNA fragmentation assays were negative for all three cell lines after a 72-h exposure to different VPA concentrations (Fig. 5A). As shown in Fig. 5B, these cell lines are able to develop late apoptotic figures visualized by DNA Hoescht staining when treated with trichostatin A. From these data we assume that apoptosis might not be the primary death mechanism and that other cell death mechanisms could be provoked by VPA.

*VPA does not induce a senescence-like phenotype in OPM2, RPMI and U266 cells*. Various cytotoxic agents have been reported to induce senescence-like phenotype (SLP) in cancer cells at low doses (24-26). However the biochemical changes associated with SLP are not clearly understood until now. We first determined the optimal condition of VPA for induction of SA-ß-gal activity, a biomarker for cellular senescence. We chose a concentration of VPA, where cells are still growing, but slower than the VPA-free control cultures. OPM2 and RPMI cultures were treated with 0.1 mM VPA for 7 days

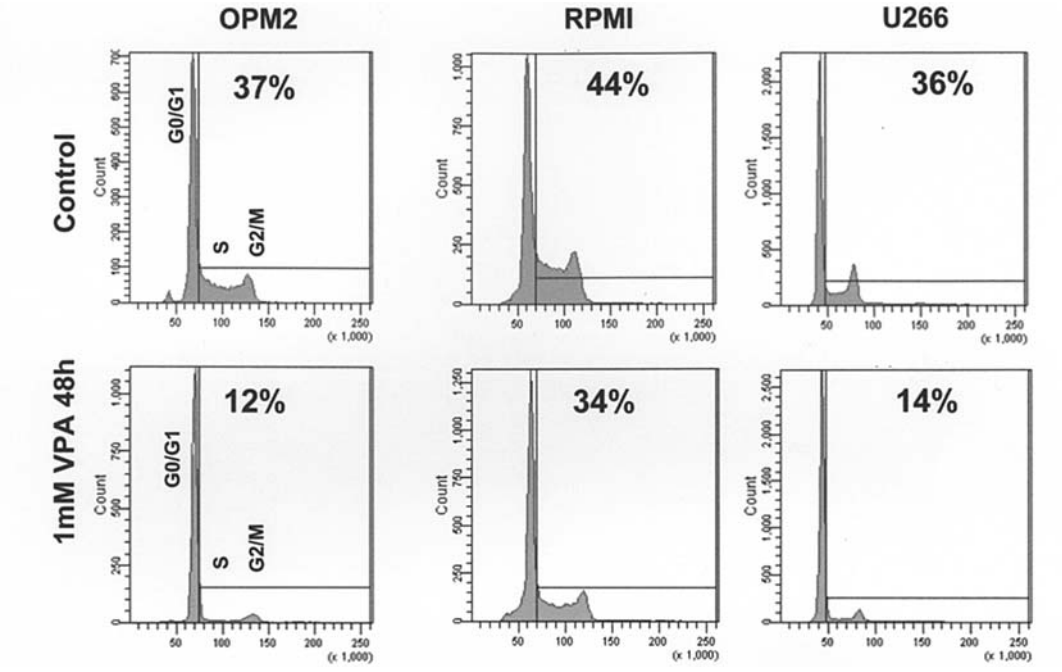
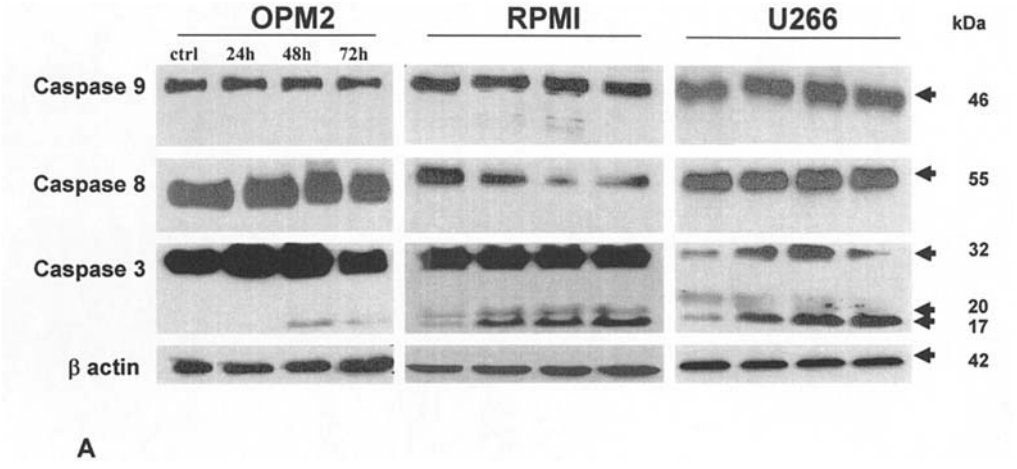



Figure 2. Effects of VPA on the cell cycle. OPM2, RPMI and U266 cells treated for 48 h with 1 mM of VPA were stained with PI and analyzed by flow cytometry. Percentages are mean values of mitotic cells representative of three independent experiments.



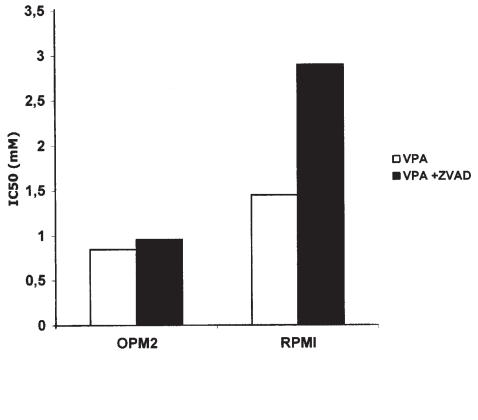
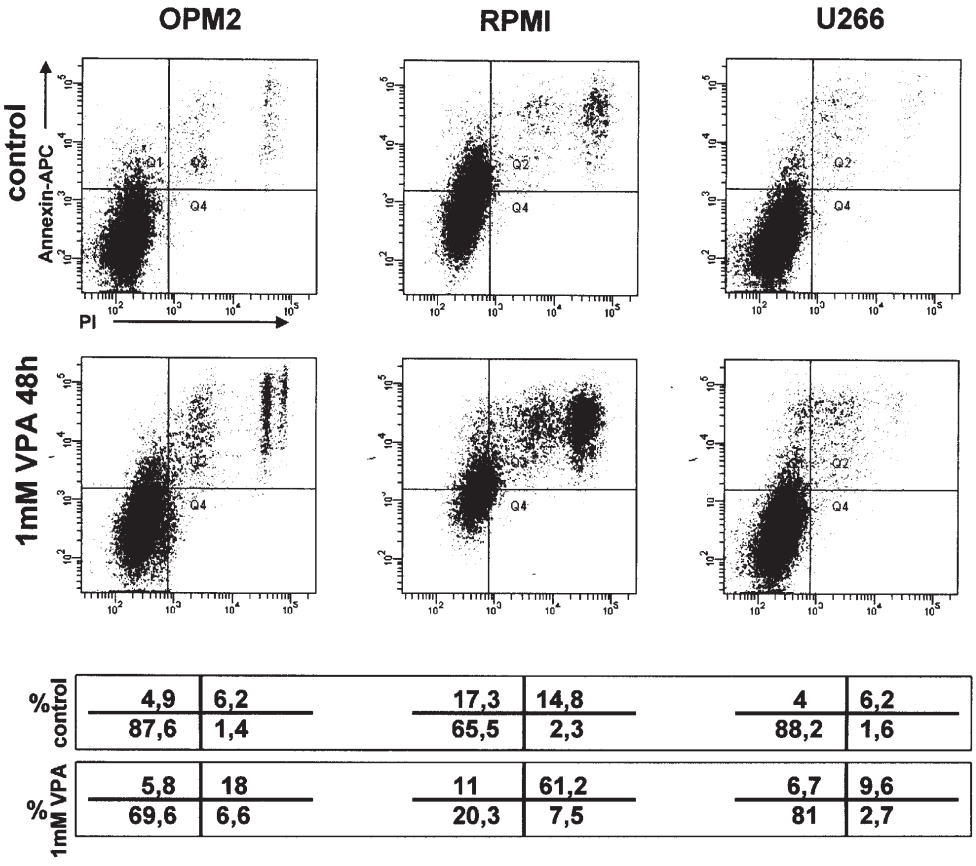



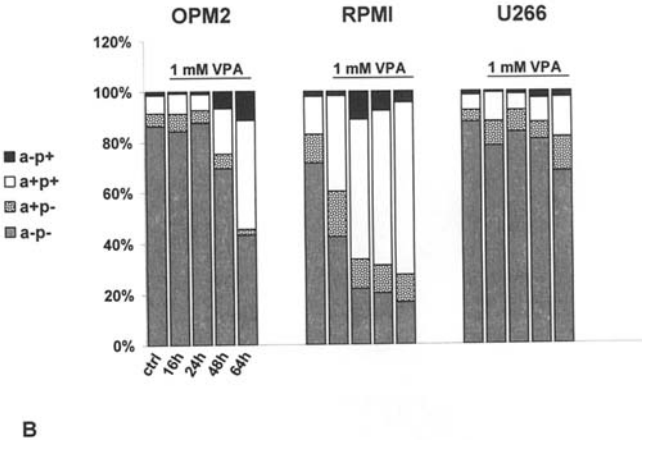

Figure 3. Effects of VPA on caspase activation. (A) OPM2, RPMI and U266 cells were treated with 1 mM of VPA during a time-course of 72 h. Controls were grown simultaneously under same conditions but without VPA. Cell aliquots were taken at the indicated time-points and protein expression of caspases 9, 8 and 3 was determined by immunoblotting as described. ß-actin was used as a loading control. Arrows indicate the specific signals. (B) OPM2, RPMI 8226 and U266 were treated with varying concentrations of VPA for 48 h with or without pancaspase inhibitor ZVAD-FMK. Cell viability was assessed by the XTT assay. VPA concentration resulting in 50% cell viability with or without 10 $\mu$M ZVAD-FMK was scored. Values represent the means from three independent experiments performed.

whereas the less sensitive cell line U266 was treated with 0.4 mM VPA. Percentages of blue positive cells expressing ß-galactosidase were calculated proportionately to the negative cells within each population by counting random fields of 100 cells each. For OPM2, we were not able to detect ß-gal expression neither in the untreated control nor in the VPA exposed cell population. In RPMI and U266 cells we observed a non-significant number of positive cells in VPA-treated and




| | | | | | | |
|---|---|---|---|---|---|---|
| % control | 4,9 | 6,2 | 17,3 | 14,8 | 4 | 6,2 |
| | 87,6 | 1,4 | 65,5 | 2,3 | 88,2 | 1,6 |
| % 1mM VPA | 5,8 | 18 | 11 | 61,2 | 6,7 | 9,6 |
| | 69,6 | 6,6 | 20,3 | 7,5 | 81 | 2,7 |

A




B

Figure 4. Detection of VPA-induced membrane changes by the annexin V/PI binding assay. (A) OPM2, RPMI and U266 cells cultured for 48 h with or without 1 mM VPA were stained with Annexin V-APC and PI and analyzed on a flow cytometer. The percentage of the cells in each quadrant indicates the mean value of three independent experiments. (B) Annexin V-APC and PI binding was monitored within the indicated time-course and time-points from OPM2, RPMI and U266 cultures exposed to 1 mM of VPA. For each time-point, the ratio of annexin V/PI-double negative (a-p-, grey), annexin V-single positive (a+p-, dotted), PI-single positive (a-p+, black) and annexin V/PI-double positive (a+p+, white) cells within the same samples are represented. Controls were cultured simultaneously under the same conditions but without VPA.

untreated populations: 0.21±0.33% and 0.14±0.23% respectively for RPMI; 1.1±1.22% and 0.8±0.55% respectively for U266. In conclusion, these results indicate that there is no VPA-induced SLP in the tested MM cell lines.

*Autophagosome formation is induced by VPA*. MDC is a weak amine that accumulates into acidic endosomal/lysosomal vacuoles. Hydrophobic interactions with lipids then enhance the fluorescence of MDC. Specificity of MDC to autophagosomes is due to the high content of unhydrolyzed membrane lipids from mitochondria and other organelles. In all three cell lines, autophagosomes were visible in the cytosol 48 h after VPA treatment (Fig. 6).

## Discussion

HDAC inhibitors have been shown to induce growth inhibition and cell death preferentially in transformed cells, making them promising cancer therapeutic agents (27,28). In the present study, we demonstrate that the short fatty acid VPA inhibits cell growth and induces cell death in multiple myeloma cell lines.

Several HDAC inhibitors have been reported already to exert cytotoxicity on MM cell lines (29,30). We demonstrated that VPA had *in vitro* cytotoxic effects on all three tested cell lines in a dose- and time-dependent manner, whereas the sensitivity of the different cell lines to VPA varied. Even

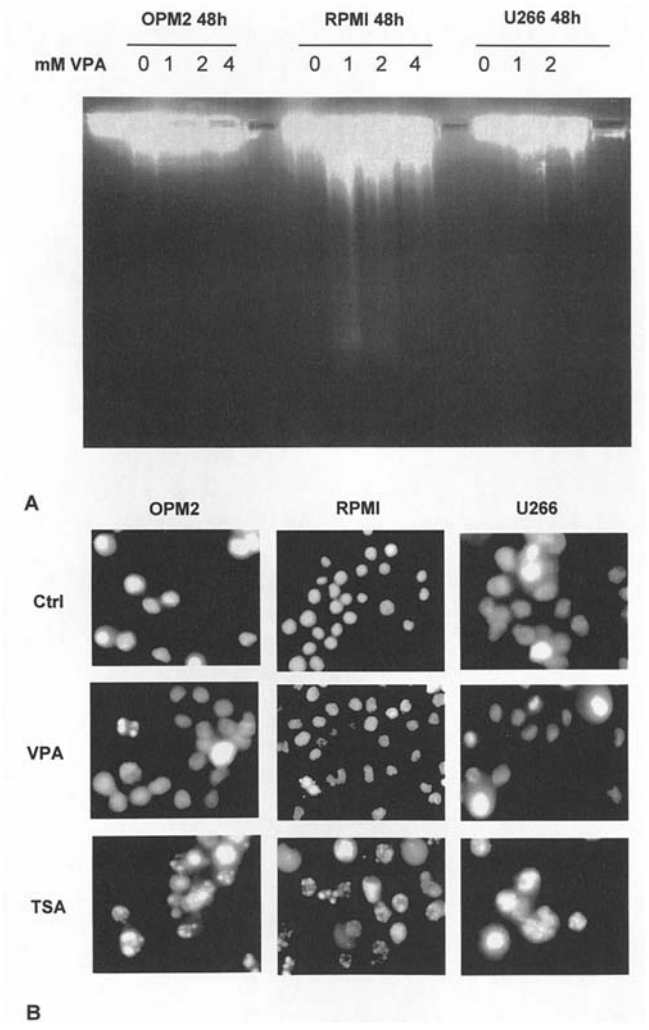



Figure 5. Apoptotic body detection and DNA laddering. (A) DNA fragmentation assay. OPM2, RPMI and U266 cell cultures were incubated for 72 h with the indicated VPA concentrations. Controls were treated in the same conditions but without VPA. (B) Hoescht staining. Cell lines were treated with valproic acid concentration 1 mM or TSA 2 $\mu$M for 48 h. After treatment, cell lines were stained with 1 $\mu$M of Hoechst 33342 solution for 1 h at 37˚C and observed by fluorescence microscopy.

within the physiological range (1-2 mM), (31,32), VPA was strongly cytotoxic on OPM2 and RPMI. Moreover, VPA-induced growth inhibition was associated with a $G_0/G_1$ arrest in all three tested cell lines. HDAC inhibitors such as sodium butyrate, retinoic acid and trichostatin A cause a cell cycle arrest in $G_0/G_1$ phase. Lavelle *et al* demonstrated that $G_1$ phase arrest is associated with an increase in CDK inhibitor $p21^{WAF}$ and a dephosphorylation of the retinoblastoma protein (RB), suggesting that the $G_1$ arrest and growth inhibition was mediated by effects on the RB pathway (29,33,34). Strikingly, similar cell accumulation values in $G_0/G_1$ phase were obtained for OPM2 and U266, although U266 cells show relatively low sensitivity to VPA. A similar effect on these cells was observed by Lavelle *et al*. After a 24-h treatment with the HDAC inhibitor sodium butyrate, RPMI and U266 accumulated similarly in $G_0/G_1$ phase but apoptotic and dead U266 cells accumulated more slowly. In this study, PARP cleavage was delayed and reduced in U266 cells. Therefore, the effects of HDAC inhibitors on cell cycle kinetics might be different to the kinetics leading to cell death among MM cell lines. Another study on HDAC expression profiles by real-time RT-PCR has identified that the greater resistance of U266 was associated with 5-10 fold increased expression of SIRT5, a single member of the sirtuin family of deacetylases (ASH Annual Meeting Abstracts, Blood 106: 5148, 2005).

HDAC inhibitors are known to induce programmed cell death in cancer cells, although the underlying mechanisms are far from being understood and seem to be very heterogeneous. Many HDAC-I-mediated death mechanisms have already been described, including induction of a senescence-like phenotype (33,34) and autophagic cell death (27,35). This diversity of cell death mechanisms was also observed for VPA (19,36,37). Recently one report demonstrated the anti-tumor activity of VPA on the MM cell lines OPM2, LP-1 and NCI-H929 (38); moreover, induction of apoptosis has been reported for these cell lines.

The phenomenon of apoptosis can be characterized by specific techniques. Externalized phosphatidylserine (PS)

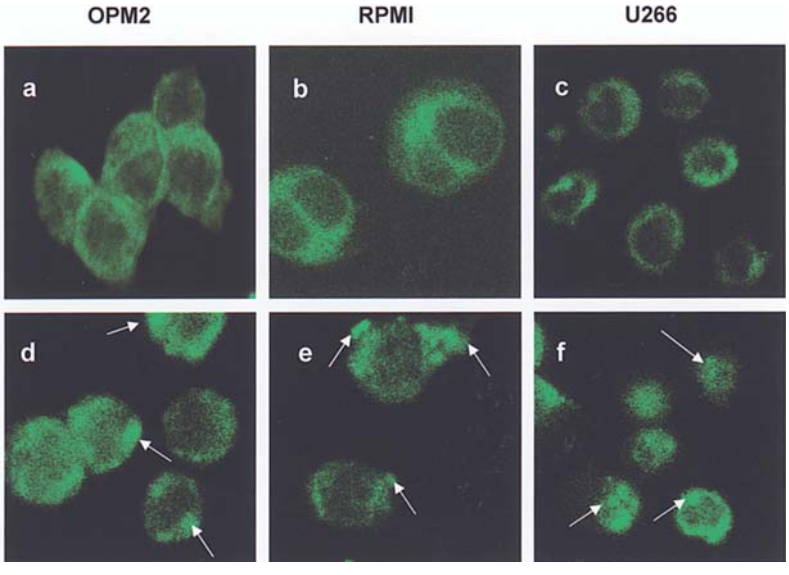



Figure 6. Fluorescence confocal microscopy for autophagosome detection. Cell lines were treated with 1 mM of VPA for 48 h. Thereafter the cell lines were treated with 100 $\mu$M MDC for 1 h at 37˚C and washed 3 times with PBS. Analysis was performed by confocal microscopy. a-c, controls; and d-f, treated cells.

on the cell surface, detected with fluorochrome-labeled annexin V by a flow cytometer is considered a hallmark of apoptosis. Annexin V-positive but PI-negative cells are defined as early apoptotic cells, whereas annexin V/PI-positive cells are considered as late apoptotic cells (39). We observed membrane changes on the MM cell lines upon VPA exposure. However, externalization of PS and plasma membrane permeability to the vital dye PI seem to occur simultaneously. From our annexin V/PI binding assays, we made the following observations: a) the amount of annexin V-single positive cells from the VPA-treated samples was not significantly different to the untreated control samples within the applied time-course; b) instead, annexin V/PI-double positive cells accumulated upon VPA treatment already at early time-points. Consequently, only the so-called late apoptotic/secondary necrotic cell number increased during VPA treatment. It is known that during necrosis, PS becomes accessible due to the disruption of membrane integrity and that phagocytosis of necrotic cells by macrophages is also PS-dependent (40). PS externalization does therefore not guarantee apoptosis in this model. Moreover, late apoptosis characterized by internucleosomal DNA fragmentation was not detected in our tested cell lines after a 72-h exposure to VPA in DNA laddering experiments.

*CD45 is a common leukocyte antigen*. The expression of this transmembrane glycoprotein on the surface of all hematopoietic cells and their precursors (except for mature erythrocytes and platelets) is quite variable. Clinical and animal studies associate CD45 expression with a phenotype linked to the progression of multiple myeloma and the susceptibility to apoptosis (41,42). Interestingly, in our study, different levels of CD45 expression were detected in the three cell lines. VPA sensitivity seems to be correlated with CD45 expression. This could explain the difference in VPA cytotoxicity between the three cell lines. The U266 cell line was positive for this marker with a low sensitivity as previously described (43). RPMI $CD45^{+/-}$ and OPM2 $CD45^{-}$ cell lines responded better to VPA treatment. Contrary to the observations by Liu, *et al* (44), CD45 is not correlated with late apoptotic cells (Annexin $V^{+}$/ $PI^{+}$) in our study.

Two different apoptotic pathways of VPA have been suggested in leukemic cells by Kawagoe *et al*; a caspase-dependent, involving the mitochondrial pathway with nuclear changes, and a caspase-independent pathway mediating events on the cell membrane (19). In our study, we prove that VPA induces processing of caspase 3 to its active forms within a time-course of 72 h in all three tested cell lines, whereas cleavage of caspases 9 and 8 was not detected in any of these cell lines. Activation of caspase 3 was delayed in OPM2 cells to 48 h in comparison to RPMI and U266 cells where it was already detectable after 24 h. This incomplete apoptotic pathway induced by VPA in these cell lines could result from a default in the MM cell machinery. However, late apoptotic figures when treated with tricostatin A and stained with DNA Hoescht staining were clearly observed. Moreover, the full apoptotic machinery to generate a complete response has previously been described in different studies on these MM cell lines treated with various agents as inhibitors of HDACs, proteasome or NFκB and etodolac. Most of these molecules induced activation of caspases 9, 8 and 3, either simultaneously or not (45-48). In accordance with our results, Pei *et al* have observed cleavage of caspase 3 by another HDAC inhibitor, sodium butyrate, when treated at 1 mM as soon as after 20 h. However, activation of caspases 9 and 8 was only induced upon sequential treatment with bortezomib/SAHA or bortezomib/sodium butyrate (45). This kinetic is not correlated with the effect of nuclear factor κ B inhibitor, 1'-acetoxychavicol acetate, which induces activity of caspases 3, 8, and 9 at 10 mM after 3 h of treatment.

The pan-caspase inhibitor ZVAD-FMK did not prevent cell death, indicating that, as observed already in leukemic cell lines, caspase-independent death mechanisms might be triggered by VPA in these MM cell lines. According to these data, we assume that nonapoptotic forms of cell death with features of necrosis and/or autophagy might be induced by VPA. Recently, one report demonstrated the anti-tumor activity of VPA on the MM cell lines OPM2 (38). These authors reported that VPA induces growth inhibition (measured by the MTT assay), cell cycle arrest in $G_0/G_1$ phase (data not shown), accumulation of acetylated H3 histone, an increase of p21 levels and a reduction in VEGF expression levels. Moreover, the extent of apoptosis has been assessed for these cell lines by the annexin V/PI binding assay. Although the authors published apoptosis quantifications without comparing untreated to treated cells, the shown data do not discriminate between the amounts of annexin V-single positive and annexin V/PI-double positive cells. Experiments implicating VPA's action on caspase activation or other apoptosis-related compounds were not performed in the mentioned report.

Other programmed cell death forms besides apoptosis such as senescence-like phenotype (SLP) and autophagic cell death are gaining more interest (49). VPA has already been reported to induce SLP in medulloblastomas (37). In ß-galactosidase assays, activation of this senescence-associated enzyme was observed after a histochemical reaction. We tested if VPA was able to induce SLP in the MM cell lines RPMI, OPM2 and U266. Our results provided no evidence for SLP in these three cell lines.

The link between caspases and autophagic cell death, opening the way for a new cell death mechanism, has recently been reviewed (50). Other studies indicate that both caspases and autophagic cell death can be simultaneously activated within a dying cell; examples exist where active caspase 3 was associated with autophagic cell death (51-53). Since we did not observe cleavage of the activator caspases 8 and 9, which typically are activated during the initiation of the intrinsic and the extrinsic apoptotic pathways respectively, but only cleavage of caspase 3, we speculate that autophagic cell death might be involved. These initial experiments elucidate autophagic cell death as a potential mechanism provoked by VPA on MM. Confocal microscopy in presence of specific fluorescent molecules showed that this phenomenon is involved in VPA-induced cytotoxicity. An improved study is underway to confirm autophagy mechanisms in this model.

**Acknowledgements**

Supported by a grant from RTL-Télévie No. 7.4.585.03F.

## References


1. Kyle RA and Rajkumar SV: Multiple myeloma. N Engl J Med 351: 1860-1873, 2004.
2. Proskuryakov SY, Konoplyannikov AG and Gabai VL: Necrosis: a specific form of programmed cell death? Exp Cell Res 283: 1-16, 2003.
3. Lockshin RA and Zakeri ZÖ: Caspase-independent cell death? Oncogene 23: 2766-2773, 2004.
4. Jaattela M and Tschopp J: Caspase-independent cell death in T lymphocytes. Nat Immunol 4: 416-423, 2003.
5. Hirsch T, Marchetti P, Susin SA, *et al*: The apoptosis-necrosis paradox. Apoptogenic proteases activated after mitochondrial permeability transition determine the mode of cell death. Oncogene 15: 1573-1581, 1997.
6. Gozuacik D and Kimchi A: Autophagy as a cell death and tumor suppressor mechanism. Oncogene 23: 2891-2906, 2004.
7. Schmitt CA: Senescence, apoptosis and therapy - cutting the lifelines of cancer. Nat Rev Cancer 3: 286-295, 2003.
8. Roninson IB, Broude EV and Chang BD: If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. Drug Resist Updat 4: 303-313, 2001.
9. Cheung P, Allis CD and Sassone-Corsi P: Signaling to chromatin through histone modifications. Cell 103: 263-271, 2000.
10. Coffey DC, Kutko MC, Glick RD, *et al*: The histone deacetylase inhibitor, CBHA, inhibits growth of human neuroblastoma xenografts *in vivo*, alone and synergistically with all-trans retinoic acid. Cancer Res 61: 3591-3594, 2001.
11. Marks PA, Richon VM, Breslow R and Rifkind RA: Histone deacetylase inhibitors as new cancer drugs. Curr Opin Oncol 13: 477-483, 2001.
12. Cinatl J Jr, Cinatl J, Driever PH, *et al*: Sodium valproate inhibits *in vivo* growth of human neuroblastoma cells. Anticancer Drugs 8: 958-963, 1997.
13. Gurvich N, Tsygankova OM, Meinkoth JL and Klein PS: Histone deacetylase is a target of valproic acid-mediated cellular differentiation. Cancer Res 64: 1079-1086, 2004.
14. Gottlicher M: Valproic acid: an old drug newly discovered as inhibitor of histone deacetylases. Ann Hematol 83 (suppl 1): S91-S92, 2004.
15. Phillips A, Bullock T and Plant N: Sodium valproate induces apoptosis in the rat hepatoma cell line, FaO. Toxicology 192: 219-227, 2003.
16. Thelen P, Schweyer S, Hemmerlein B, Wuttke W, Seseke F and Ringert RH: Expressional changes after histone deacetylase inhibition by valproic acid in LNCaP human prostate cancer cells. Int J Oncol 24: 25-31, 2004.
17. Tittle TV, Schaumann BA, Rainey JE and Taylor K: Segregation of the growth slowing effects of valproic acid from phenytoin and carbamazepine on lymphoid tumor cells. Life Sci 50: PL79-PL83, 1992.
18. Tang R, Faussat AM, Majdak P, *et al*: Valproic acid inhibits proliferation and induces apoptosis in acute myeloid leukemia cells expressing P-gp and MRP1. Leukemia 18: 1246-1251, 2004.
19. Kawagoe R, Kawagoe H and Sano K: Valproic acid induces apoptosis in human leukemia cells by stimulating both caspase-dependent and -independent apoptotic signaling pathways. Leuk Res 26: 495-502, 2002.
20. Roehm NW, Rodgers GH, Hatfield SM and Glasebrook AL: An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J Immunol Methods 142: 257-265, 1991.
21. Dimri GP, Lee X, Basile G, *et al*: A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. Proc Natl Acad Sci USA 92: 9363-9367, 1995.
22. McCarthy RC and Fetterhoff TJ: Issues for quality assurance in clinical flow cytometry. Arch Pathol Lab Med 113: 658-666, 1989.
23. Martin SJ, Reutelingsperger CP, McGahon AJ, *et al*: Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med 182: 1545-1556, 1995.
24. Chang BD, Xuan Y, Broude EV, *et al*: Role of p53 and p21waf1/cip1 in senescence-like terminal proliferation arrest induced in human tumor cells by chemotherapeutic drugs. Oncogene 18: 4808-4818, 1999.
25. Chang BD, Broude EV, Dokmanovic M, *et al*: A senescence-like phenotype distinguishes tumor cells that undergo terminal proliferation arrest after exposure to anticancer agents. Cancer Res 59: 3761-3767, 1999.
26. Eom YW, Kim MA, Park SS, *et al*: Two distinct modes of cell death induced by doxorubicin: apoptosis and cell death through mitotic catastrophe accompanied by senescence-like phenotype. Oncogene 24: 4765-4777, 2005.
27. Shao Y, Gao Z, Marks PA and Jiang X: Apoptotic and autophagic cell death induced by histone deacetylase inhibitors. Proc Natl Acad Sci USA 101: 18030-18035, 2004.
28. Insinga A, Minucci S and Pelicci PG: Mechanisms of selective anticancer action of histone deacetylase inhibitors. Cell Cycle 4: 741-743, 2005.
29. Chen YH, Lavelle D, De Simone J, Uddin S, Platanias LC and Hankewych M: Growth inhibition of a human myeloma cell line by all-trans retinoic acid is not mediated through downregulation of interleukin-6 receptors but through upregulation of p21(WAF1). Blood 94: 251-259, 1999.
30. Lavelle D, Chen YH, Hankewych M and De Simone J: Histone deacetylase inhibitors increase p21(WAF1) and induce apoptosis of human myeloma cell lines independent of decreased IL-6 receptor expression. Am J Hematol 68: 170-178, 2001.
31. Gottlicher M, Minucci S, Zhu P, *et al*: Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 20: 6969-6978, 2001.
32. Warrell RP Jr, He LZ, Richon V, Calleja E and Pandolfi PP: Therapeutic targeting of transcription in acute promyelocytic leukemia brabley use of an inhibitor of histone deacetylase. J Natl Cancer Inst 90: 1621-1625, 1998.
33. Place RF, Noonan EJ and Giardina C: HDACs and the senescent phenotype of WI-38 cells. BMC Cell Biol 6: 37, 2005.
34. Xu WS, Perez G, Ngo L, Gui CY and Marks PA: Induction of polyploidy by histone deacetylase inhibitor: a pathway for anti-tumor effects. Cancer Res 65: 7832-7839, 2005.
35. Marks PA and Dokmanovic M: Histone deacetylase inhibitors: discovery and development as anticancer agents. Expert Opin Investig Drugs 14: 1497-1511, 2005.
36. Insinga A, Monestiroli S, Ronzoni S, *et al*: Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway. Nat Med 11: 71-76, 2005.
37. Li XN, Shu Q, Su JM, Perlaky L, Blaney SM and Lau CC: Valproic acid induces growth arrest, apoptosis, and senescence in medulloblastomas by increasing histone hyperacetylation and regulating expression of p21Cip1, CDK4, and CMYC. Mol Cancer Ther 4: 1912-1922, 2005.
38. Kaiser M, Zavrski I, Sterz J, *et al*: The effects of the histone deacetylase inhibitor valproic acid on cell cycle, growth suppression and apoptosis in multiple myeloma. Haematologica 91: 248-251, 2006.
39. Bossy-Wetzel E and Green DR: Detection of apoptosis by annexin V labeling. Methods Enzymol 322: 15-18, 2000.
40. Brouckaert G, Kalai M, Krysko DV, *et al*: Phagocytosis of necrotic cells by macrophages is phosphatidylserine dependent and does not induce inflammatory cytokine production. Mol Biol Cell 15: 1089-1100, 2004.
41. Ishikawa H, Tsuyama N, Liu S, *et al*: Accelerated proliferation of myeloma cells by interleukin-6 cooperating with fibroblast growth factor receptor 3-mediated signals. Oncogene 24: 6328-6332, 2005.
42. Moreau P, Robillard N, Avet-Loiseau H, *et al*: Patients with CD45 negative multiple myeloma receiving high-dose therapy have a shorter survival than those with CD45 positive multiple myeloma. Haematologica 89: 547-551, 2004.
43. Asosingh K, De Raeve H, Menu E, *et al*: Angiogenic switch during 5T2MM murine myeloma tumorigenesis: role of CD45 heterogeneity. Blood 103: 3131-3137, 2004.
44. Liu S, Ishikawa H, Tsuyama N, *et al*: Increased susceptibility to apoptosis in CD45(+) myeloma cells accompanied by the increased expression of VDAC1. Oncogene 25: 419-429, 2006.
45. Pei XY, Dai Y and Grant S: Synergistic induction of oxidative injury and apoptosis in human multiple myeloma cells by the proteasome inhibitor bortezomib and histone deacetylase inhibitors. Clin Cancer Res 10: 3839-3852, 2004.
46. Ito K, Nakazato T, Xian MJ, *et al*: 1'-acetoxychavicol acetate is a novel nuclear factor kappaB inhibitor with significant activity against multiple myeloma *in vitro* and *in vivo*. Cancer Res 65: 4417-4424, 2005.

47. Nakamura S, Kobayashi M, Shibata K, *et al*: Etodolac induces apoptosis and inhibits cell adhesion to bone marrow stromal cells in human myeloma cells. Leuk Res 30: 123-135, 2006.
48. Hideshima T, Catley L, Yasui H, *et al*: Perifosine, an oral bioactive novel alkylphospholipid, inhibits Akt and induces *in vitro* and *in vivo* cytotoxicity in human multiple myeloma cells. Blood 107: 4053-4062, 2006.
49. Edinger AL and Thompson CB: Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol 16: 663-669, 2004.
50. Yu L, Lenardo MJ and Baehrecke EH: Autophagy and caspases: a new cell death program. Cell Cycle 3: 1124-1126, 2004.
51. Mills KR, Reginato M, Debnath J, Queenan B and Brugge JS: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is required for induction of autophagy during lumen formation *in vitro*. Proc Natl Acad Sci USA 101: 3438-3443, 2004.
52. Martin DN and Baehrecke EH: Caspases function in autophagic programmed cell death in *Drosophila*. Development 131: 275-284, 2004.
53. Lee CY and Baehrecke EH: Steroid regulation of autophagic programmed cell death during development. Development 128: 1443-1455, 2001.