Malignant pleural mesothelioma is a neoplastic disease of the pleura strongly associated with exposure to asbestos fibers (1). Mesothelioma is classified into three major histologic subtypes: epithelioid (50% of cases), sarcomatoid (15% of cases), and biphasic or mixed (35% of cases). Mesothelioma has a strong male predominance and is usually diagnosed in the fifth to seventh decade of life, 30 to 40 years after the occupational exposure (2), reflecting the past production and use of asbestos in industry (3, 4). Currently, there is no curative treatment for malignant pleural mesothelioma, tumor cells being particularly resistant to chemotherapy, the main actual treatment for these patients. Indeed, malignant pleural mesothelioma is refractory to most of the common tumor-directed therapies with a median survival of about 8 to 12 months (5) and a response rate <20% (6). To date, the standard treatment used in first line for malignant pleural mesothelioma is chemotherapy based on an association of cisplatin and pemetrexed. Cisplatin (cis-diamminedichloridoplatinum) is a platinum-based chemotherapeutic drug belonging to the DNA-damaging agents, mainly forming covalent links with DNA (7). Pemetrexed is a multitarget antifolate agent active on three enzymes involved in synthesis of thymidine and purine nucleotides: thymidilate synthetase, dihydrofolate reductase, and glycinamide ribonucleotide formyl-transferase (8, 9). The cisplatin-pemetrexed combination yielded the best effect for malignant pleural mesothelioma in terms of median survival (12.1 months), median time to disease progression (5.7 months), and response rate (41%) in a phase III study (10). Nevertheless, the major concerns of this treatment are short duration of response, rapid relapse, and acute toxicities, some of them being prevented by supplementation of folic acid and vitamin B12 (11).

## Abstract

**Purpose:** Present chemotherapeutic regimens are marginally efficient in tumor cells being particularly resistant to radiotherapy and/or chemotherapy. We hypothesized that unresponsiveness of tumors to conventional therapeutic agents might be due to inappropriate gene expression resulting from epigenetic modifications and leading to transcriptional silencing. The goal of this study was to evaluate the anticancer effect of a histone deacetylase inhibitor, valproate, on mesothelioma cells in combination with pemetrexed and cisplatin, the usual first-line regimen of chemotherapy for this tumor.

**Experimental Design and Results:** We show that valproate augments apoptosis induced by pemetrexed and cisplatin in mesothelioma cell lines and in tumor cells from patient’s biopsies. Onset of apoptosis involves both extrinsic and intrinsic pathways requiring enzymatic activities of caspases 8 and 9, respectively. Valproate but not suberoylanilide hydroxamic acid efficiently stimulates the production of reactive oxygen species. The free radical scavenger N-acetylcysteine inhibits apoptosis, indicating that reactive oxygen species are major mediators of valproate activity. As expected, valproate alone or combined with pemetrexed and cisplatin triggers hyperacetylation of histone H3. Bid protein processing in truncated t-Bid and cytochrome c release from mitochondria are significantly increased in the presence of valproate, providing a mechanistic rationale for improvement of the proapoptotic efficacy of cisplatin and pemetrexed. Finally, valproate when combined with pemetrexed and cisplatin prevents tumor growth in mouse models of epithelioid mesothelioma.

**Conclusions:** These observations support the potential additional efficacy of valproate in combination with pemetrexed and cisplatin for treatment of malignant mesothelioma.
Recent evidence indicates that modulation of gene expression by epigenetic modifications is an important parameter of cancer progression (12, 13). We hypothesized that the unresponsiveness of tumors to conventional therapeutic agents might be due to inappropriate gene expression leading to transcriptional silencing. Histone acetylation, one of the most important epigenetic modifications, is regulated by two families of enzymes: histone acetyltransferases and histone deacetylases (HDAC) adding and removing acetyl groups on lysine residues of histone, respectively. Acetyl removal by HDACs restores a positive charge to the lysine residues in the histone N-terminal tails and is thought to increase the affinity of histones for DNA, leading to DNA condensation and transcriptional repression (14–16). A proposed mechanism for the antitumor effects of HDACs inhibitors is the accumulation of acetylated histones leading to transcriptional activation of a selected number of genes whose expression causes inhibition of tumor cell growth and induction of apoptosis (17). Among HDAC inhibitors, valproate (the sodium salt of 2-propylpentanoic acid) has the major advantage of being commonly prescribed as an antiepileptic drug. Its pharmacokinetics and toxicity profiles are thus well documented (18–20). In vitro, valproate induces differentiation, growth arrest, and/or apoptosis in a broad spectrum of transformed cells through HDAC inhibition (21, 22). For example, valproate potentiates the cytotoxic effect of tumor necrosis factor–related apoptosis-inducing ligand on cultured thoracic cancer cells through mitochondria-dependent caspase activation (23). Another HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), modulates expression of genes involved in cell cycle control, differentiation, and apoptosis (24). SAHA was recently evaluated in a clinical trial for treatment of mesothelioma (25) and a randomized phase III study is now open.

In this report, we evaluated the potency of valproate to improve the antitumor efficacy of cisplatin and pemetrexed in malignant pleural mesothelioma cell cultures as well as in tumor xenograft mouse models.

**Materials and Methods**

**Cell culture and drugs.** The human mesothelioma cell lines M14K, M38K, and ZL34 belong to the epithelioid, biphasic, and sarcomatoid subtypes, respectively. AB12 is a mesothelioma cell line of murine origin. Cells were cultivated in RPMI 1640 medium supplemented with 10% FCS, 2 mmol/L L-glutamin, 100 U/mL of penicillin, and 100 μg/mL of streptomycin, and maintained at 37°C in a humidified atmosphere with 5% CO2. Cells were incubated with 2 mmol/L valproate (Sigma Aldrich), 2 μmol/L SAHA (Alexis), 10 μmol/L cisplatin (Bristol-Myers Squibb), and 200 μmol/L pemetrexed (Lilly) alone or in combination. All the compounds were solubilized in water except for SAHA that was diluted in DMSO. Of note, the final concentration of DMSO in the culture medium never exceeded 0.1% and did not affect results (data not shown). Untreated cells were used as controls.

**Cell viability assay.** As a surrogate of growth, cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 Aqueous One Solution Cell Proliferation assay; Promega). Mesothelioma cell lines (1.10^6 cells/mL) were incubated with different concentrations of valproate (0-10 mmol/L), SAHA (0-10 μmol/L), cisplatin (0-100 μmol/L), or pemetrexed (0-5 mmol/L). After 24 h of culture, 20 μL of tetrazolium-containing reagent were added to each well in a 96-wells plate. After 2 h incubation at 37°C, the plate was analyzed using a colorimetric microplate reader at a wavelength of 490 nm.

**Detection of apoptosis.** Apoptosis was quantified using the Annexin V-phycocerythrin apoptosis detection kit (Becton Dickinson), which labels phosphatidylserine externalized in the early phases of apoptosis. Cells were plated at 1.10^4 per mL in 24-wells plates and treated with 2 mmol/L valproate, 2 μmol/L SAHA, 10 μmol/L cisplatin, and 200 μmol/L pemetrexed alone or in combination. After 24 h of culture, floating and adherent cells were combined, washed twice with cold PBS, resuspended in 100 μL of annexin binding buffer (10 mmol/L Heps, 140 mmol/L NaCl, 2.5 mmol/L CaCl2, pH 7.4), incubated for 15 min at room temperature with 5 μL of Annexin V-phycocerythrin and 5 μL of 7AAD and analyzed by flow cytometry (FACS Aria; Becton Dickinson). Ten thousand events were collected and analyzed with the FACS Diva Software. An-positive/7AAD-negative and An-positive/7AAD-positive cells were considered as early and late apoptotic, respectively.

To assess the role of caspases in apoptotic pathways, 1.10^5 cells per mL were incubated with or without 20 μmol/L of total pancaspase inhibitor Z-Val-Ala-Asp(OMe)-CH2F (Z-VAD-fmk; Becton Dickinson), 20 μmol/L of negative control (Z-FA-fmk; Becton Dickinson), 50 μmol/L caspase 8 specific inhibitor Z-Ile-Glu(Ome)-Thr-Asp(Ome)-CH2F (Z-IETD-fmk; Calbiochem), or 50 μmol/L caspase 9 specific inhibitor Z-Leu-Glu(Ome)-His-Asp(Ome)-CH2F (Z-LEHD-fmk; Calbiochem), all being diluted in DMSO.

**Cell cycle analysis.** Cell cycle was analyzed by flow cytometry after 48 h of culture in the presence of 2 mmol/L valproate, 2 μmol/L SAHA, 10 μmol/L cisplatin, and 200 μmol/L pemetrexed alone or in combination. Briefly, 5.10^5 cells were trypsinized, collected by centrifugation at 500 g for 10 min, washed twice with PBS-10% FCS, and fixed with 70% cold ethanol. After incubation at -20°C for at least 1 h, cells were washed twice and treated for 30 min at 37°C with RNase A (50 μg/mL; Sigma Aldrich). Then, cells were incubated for 10 min in the dark in PBS containing 20 μg/mL propidium iodide (Sigma Aldrich) and analyzed by flow cytometry (FACS Aria; Becton Dickinson). Cell doubllets were excluded from the analysis using the (FSC-H/FSC-W) gating method. Ten thousand events were collected and analyzed with the FACS Diva Software.

**Western blot analysis.** After 24 h of culture, 1.10^6 cells were washed twice in cold PBS and lysed on ice for 30 min in 50 mmol/L Tris-HCl (pH 7.5), 1% NP-40, 0.5% sodium deoxycholate, 150 mmol/L NaCl, and 0.1% SDS supplemented with a protease inhibitor cocktail (Complete; Roche). After centrifugation at 13,000 g for 5 min at 4°C, the supernatants containing soluble proteins were collected and stored at -80°C. To analyze cytoschrome c and Bax expression, cytosolic and mitochondrial fractions were prepared using the Mitochondria Extrac- tion Kit (Pierce) and stored at -80°C. Protein concentrations were quantified using the Micro BCA Protein Assay kit (Pierce). Twenty micrograms of proteins were migrated on a 15% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (GE Healthcare). After blocking with TBS containing 5% of non-fat dry milk, the membrane was incubated overnight at 4°C with primary antibodies.
antiacetylated histone H3 (dilution 1/10,000; Upstate), anti-actin (dilution 1/1,000; Sigma Aldrich), anti-Bax (dilution 1/250; Dako Cytomation), anti-p21 (dilution 1/500; Santa Cruz Biotechnology), anti-cyclin D1 (dilution 1/1,000; Sigma Aldrich), anti-Bid (dilution 1/500; Becton Dickinson), anti-PCNA (dilution 1/500; Upstate), anti-Bcl-2 (dilution 1/100; Dako Cytomation), anti-Bcl-X, (dilution 1/200; Sigma Aldrich), anti-p53 (dilution 1/1,000; Becton Dickinson Pharmingen), anti-Erk (dilution 1/10,000; Sigma Aldrich), anti-phospho-Erk (dilution 1/1,000; Cell Signalling) and anti-VDAC1 (dilution 1/100; Abcam). Optimal antibody concentrations were provided by the manufacturer and tested experimentally. Primary antibodies were then incubated for 1 h with 10,000-fold diluted polyclonal horseradish peroxidase–conjugated secondary antibodies (either goat antimouse or swine antirabbit immunoglobulin/horseradish peroxidase purchased from Dako Cytomation) and revealed using the ECL Advance Western Blotting Detection kit (GE Healthcare).

Measurement of reactive oxygen species production. Reactive oxygen species (ROS) production was detected using 5,6-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA; Invitrogen). After 30 min of incubation with 5 μmol/L of CM-H2DCFDA, the different compounds (valproate, SAHA, cisplatin, and pemetrexed) were added alone or in combination. After 24 h of culture, mesothelioma cell lines (1.106 cells per ml in a 24-well plate) were harvested, washed with PBS, and analyzed by flow cytometry (FACS Aria; Becton Dickinson). ROS production was quantified using the fluorescence intensity of chloromethyldichlorofluorescein. Ten thousand events were collected and analyzed with the FACS Diva Software. Cells were also treated with 100 μmol/L hydrogen peroxide (H2O2), or 10 mmol/L of the free radical scavenger N-acetylcysteine (Calbiochem) as positive and negative controls, respectively.

Evaluation of regimen efficacy in SCID mice. BALB/c Han-Hsd-Pkrdc SCID mice (Harlan Nederland, NM Horst) received a standard research diet supplemented with vitamin B12 (165 mg/kg; Sigma) throughout the experiment. Two million of M14K and ZL34, embedded in 50% Matrigel Basement Membrane Matrix High Concentration (Becton Dickinson Biosciences), were implanted s.c. in each flank of 7 week-old SCID mice (Harlan Nederland, NM Horst) received a standard research diet supplemented with vitamin B12 (165 mg/kg; Sigma) throughout the experiment. Two million of M14K and ZL34, embedded in 50% Matrigel Basement Membrane Matrix High Concentration (Becton Dickinson Biosciences), were implanted s.c. in each flank of 7 week-old SCID mice. Similarly, AB12 cells were injected s.c. in BALB/c mice. When tumors reached a volume of 300 to 400 mm3, the mice were administered with daily i.p. injections of valproate (400 mg/kg/d), SAHA (50 mg/kg/d), or PBS as control. Three days after HDAC inhibitor administration, a single i.p. injection of cisplatin (2 mg/kg) and daily injections of pemetrexed (30 mg/kg/d) were done. The schedule of drugs injections was first determined in preliminary dose response experiments. Tumor volumes were calculated weekly using the formula: 4/3π × d (diameter)2. Groups of at least 6 mice were tested in each experimental condition.

Statistical analysis. All cell culture experiments were conducted at least three times and data are shown as means ± SD. Statistical significance was calculated using the paired Student’s t test and data were considered statistically significant (*), very statistically significant (**), and highly statistically significant (***)) at P < 0.05, P < 0.01, and P < 0.001, respectively. To determine whether the effect on apoptosis induced by the combined treatment was additive, synergistic, or antagonistic, a Synergy Index (SI) was calculated using the following formula: SI = specific apoptosis upon combined treatment / sum of specific apoptosis of single agent treatment. The percentage of specific apoptosis was determined using the following formula: specific apoptosis = (drug-induced apoptosis–spontaneous apoptosis)/(100–spontaneous apoptosis) × 100%. When SI > 1, SI = 1 and SI < 1, the effects were defined as synergistic, additive, and antagonistic, respectively.

Results

Valproate combined with cisplatin and pemetrexed affects malignant pleural mesothelioma cell viability, proliferation, and apoptosis. The standard chemotherapy of mesothelioma (i.e. pemetrexed associated with cisplatin; ref. 10) unfortunately yields low response rate (maximum 41%) and frequent relapses. Hypothesizing that the efficacy of this regimen was hampered by epigenetic mechanisms, we evaluated the potential therapeutic effect of HDAC inhibitors in cell culture. We first measured the cytotoxicity of two HDAC inhibitors (valproate and SAHA), cisplatin, and pemetrexed in three cell lines pertaining to the major histologic subtypes of mesothelioma (epithelioid, biphasic, and sarcomatoid for M14K, M38K, and ZL34, respectively). Cells were cultivated for 24 hours in the presence of increasing concentrations of valproate (0-10 mmol/L), SAHA (0-10 μmol/L), cisplatin (0-100 μmol/L), or pemetrexed (0-5 mmol/L), and their viability was estimated using a MTS assay. In all three cell lines, these compounds induced a dose-dependent decrease in cell viability with a particular cytotoxicity of >2 mmol/L for valproate, 5 μmol/L for SAHA, 10 μmol/L for cisplatin, and 1 mmol/L for pemetrexed (Fig. 1A to D, respectively).

In view of evidence that valproate reduces viability, we sought to determine whether combination with pemetrexed, cisplatin, and valproate (or SAHA) would also increase the sensitivity of mesothelioma cells to apoptosis. To unravel a synergism between two chemotherapeutic molecules, each compound should be minimally toxic. Therefore, a physiologically relevant concentration of 2 mmol/L of valproate (ref. 23; or 2 μmol/L of SAHA; ref. 26) was combined to slightly cytotoxic doses of cisplatin (10 μmol/L) and pemetrexed (200 μmol/L). Importantly, these concentrations are also within a dose range that can be achieved for chemotherapy of malignant pleural mesothelioma patients. M14K, M38K, and ZL34 cells were cultivated for 24 hours in the presence of subcytotoxic concentrations of valproate, SAHA, cisplatin, and pemetrexed alone or in combination. Early onset of apoptosis was assessed by flow cytometry after Annexin V-7AAD labeling allowing detection of phosphatidylserine externalization and loss of membrane integrity. At the concentrations tested, valproate increased apoptosis induced by cisplatin combined to pemetrexed (P < 0.05, P < 0.05, and P < 0.01 for M14K, M38K, and ZL34 respectively, according to the paired Student’s t test; Fig. 2A). Similarly, SAHA also increased cisplatin plus pemetrexed–induced apoptosis in the three cell lines (P < 0.01, according to the paired Student’s t test). The combined effect of valproate or SAHA with cisplatin and pemetrexed was synergistic when compounds were added sequentially whereas concomitant treatment yielded an additive effect (data not shown).

DNA cleavage is a late hallmark of apoptosis that can be estimated by flow cytometry after cell permeabilization and propidium iodide labeling. Cells staining in sub-G1 have lost cleaved DNA fragments and are considered to be apoptotic. The percentages of sub-G1 cells determined at 48 hours were statistically significantly increased when valproate or SAHA was combined with pemetrexed and cisplatin (black bar on Fig. 2B; P < 0.05, P < 0.01, and P < 0.05 for M14K, M38K, and ZL34, respectively, using the paired Student’s t test). Late (propidium iodine staining) and early (Annexin V) markers thus show that valproate and SAHA augment pemetrexed plus cisplatin–induced apoptosis in mesothelioma cells. Flow cytometric analysis of propidium iodide–stained cells further showed that the percentages of cells in S-G2/M phase were significantly reduced when the M14K and M38K cell lines were cultivated in presence of HDAC inhibitor compared with cisplatin and...
Taken together, these results show that valproate and SAHA synergize with cisplatin and pemetrexed to induce apoptosis and affect the percentages of cells in S-G2/M phases.

**Apoptosis induced by valproate combined to cisplatin and pemetrexed is caspase-dependent.** Onset of apoptosis occurs through extrinsic (receptor dependent) or intrinsic (mitochondrial) pathways involving caspase 8 and caspase 9 enzymatic activity, respectively. We first evaluated the implication of caspases in the mechanism of apoptosis initiated by the association of valproate (or SAHA) with cisplatin and pemetrexed using a pan caspase inhibitor (Z-VAD-fmk). Preincubation of mesothelioma cell lines with 20 μmol/L of Z-VAD-fmk for 1 hour inhibited cell death in the three cell lines ($P < 0.05$; Fig. 3). As control, we used the Z-FA-fmk analog devoid of caspase inhibition activity. We conclude that apoptosis induced by HDAC inhibitors plus cisplatin plus pemetrexed is caspase-dependent. We next selectively blocked the extrinsic or intrinsic pathways using caspase 8 (Z-IETD-fmk) and caspase 9 inhibitors (Z-LEHD-fmk), respectively. The caspase 8 inhibitor significantly decreased apoptosis in all three cell lines ($P < 0.05$). In contrast, the caspase 9 inhibitor only yielded a statistically significant decrease in ZL34 ($P < 0.05$) although a similar trend was observed in M38K cells.

These results show that apoptosis induced by valproate combined to cisplatin and pemetrexed is caspase-dependent and involves both extrinsic and intrinsic pathways.

**Valproate induces histone H3 hyperacetylation, p21 overexpression, Bid cleavage, and cytochrome c release from mitochondria.** To further understand the mechanisms involved, the expression profile of selected proteins was analyzed after 24 hours of culture in presence of different combinations of valproate (2 mmol/L and 10 mmol/L), SAHA (2 μmol/L), cisplatin (10 μmol/L), and pemetrexed (200 μmol/L). As control, protein levels in cell lysates were first normalized by Western blot using an anti-actin antibody (Fig. 4). As expected for HDAC inhibitors, valproate and SAHA induced hyperacetylation of histone H3 when added alone or in combination with cisplatin and pemetrexed. p21 was upregulated in most conditions except in the presence of pemetrexed alone. Compared with control conditions, cyclin D1 and Bcl-2 were down-regulated when valproate or SAHA were combined to pemetrexed and cisplatin (compare 0 with C+P+V and with C+P+S in Fig. 4). In contrast, PCNA, Bcl-XL and cytosolic Bax only fluctuated moderately whereas Erk/phospho-Erk remained unchanged. Importantly, the cleaved form of Bid (t-Bid) was clearly detected in the presence of valproate alone but also in combination with C+P. Finally, cytochrome c release from mitochondria into the cytoplasm was stimulated in all conditions.

Protein expression analyses show thus that valproate and SAHA induce histone H3 hyperacetylation, p21 overexpression, Bid cleavage, and cytochrome c release from mitochondria.

**Valproate induces ROS production and scavenging of free radicals inhibits apoptosis.** Because ROS are potential mediators of mitochondrial-dependent apoptosis (27), we measured ROS production in the presence of valproate combined with cisplatin and pemetrexed. When cells were treated for 24 hours with valproate alone, a significant increase of ROS production was observed ($P < 0.05$, $P < 0.05$, and $P < 0.01$ for M14K, M38K, and ZL34, respectively, according to the paired Student’s t test, Fig. 5). When valproate was combined with cisplatin and pemetrexed alone (grey bar on Fig. 2B). In ZL34 cells, treatment with cisplatin and pemetrexed increased the number of cells in S-G2/M phase compared with the control and administration of valproate (or SAHA) restored basal levels of ZL34 cells in S-G2/M.

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**Figure 1.** Effect of valproate, SAHA, cisplatin, and pemetrexed on viability of mesothelioma cell lines. Three mesothelioma cell lines (M14K, M38K, and ZL34) were cultivated in the presence of valproate (0, 0.2, 0.5, 1.2, 5, and 10 μmol/L, A), SAHA (0, 0.5, 1, 2, 5, and 10 μmol/L, B), cisplatin (0, 1, 10, 25, 50, 75, and 100 μmol/L, C), or pemetrexed (0, 10, 50, 100, 200, 1,000, and 5,000 μmol/L, D). After 24 h of culture, cell viability was estimated using the MTS assay and the measured optical densities were normalized to the control.
pemetrexed, the levels of ROS further increased and reached levels of the positive control (hydrogen peroxide: 100 μmol/L H₂O₂). In contrast, SAHA did not stimulate ROS production under the same experimental conditions even when combined with cisplatin and pemetrexed.

To assess any association between ROS production and apoptosis, cells were cultivated in the presence of 10 mmol/L N-acetylcysteine, used as ROS scavenger. In cells treated with N-acetylcysteine, a statistically significant inhibition of apoptosis was observed in all but one condition (P < 0.05; Fig. 5B). Of note, when ROS scavenging was inefficient as observed in ZL34 cells cultivated with pemetrexed, cisplatin, and valproate, the apoptotic rates also remained unchanged.

Collectively, these data indicate that ROS production is a major event in the mechanism of apoptosis induced by valproate, cisplatin, and pemetrexed.

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Fig. 2. Apoptosis and cell cycle alterations induced by valproate in combination with pemetrexed and cisplatin. Three mesothelioma cell lines (M14K, M38K, and ZL34) were cultivated for 24 h in the presence of a combination of 2 mmol/L valproate (V), 2 μmol/L SAHA (S), 200 μmol/L pemetrexed (P), and 10 μmol/L cisplatin (C). Apoptosis was evaluated by flow cytometry after Annexin V-7AAD labeling (A). The An-positive/7AAD-negative and An-positive/7AAD-positive cells were considered as early and late apoptotic, respectively. The results are presented as means and SD of three independent experiments. * and ** denote the statistically significant differences according to the paired Student’s t test P < 0.05 and P < 0.01, respectively. After 48 h of culture, cells were fixed with 70% ice-cold ethanol and labeled with propidium iodide (B). Sub-G₁ cells were considered to be apoptotic. The results are the means of three independent experiments. * and ** denote the statistically significant differences according to the paired Student’s t test P < 0.05 and P < 0.01, respectively.
Valproate prevents tumors growth in SCID mice. We next assessed the capacity of valproate and SAHA to inhibit tumor growth in mice engrafted with mesothelioma cells. In pilot dose response experiments, we found that concentrations of 400 mg/kg/day of valproate, 50 mg/kg/day of SAHA, 30 mg/kg/day of pemetrexed, and 2 mg/kg of cisplatin (single injection) were optimal dosage (data not shown). Indeed, drugs used alone in these concentrations did not show tumor growth inhibition or toxicities. The M14K, ZL34, and AB12 cell lines consistently yielded tumors in all engrafted mice. When tumors reached a volume of 300 to 400 mm³, the mice were injected i.p. with a single dose of 2 mg/kg of cisplatin and daily with 30 mg/kg of pemetrexed. This suboptimal treatment did not impair tumor progression in M14K xenografted mice (compare C+P and control PBS in Fig. 6). In this model, 400 mg/kg/day of valproate alone was unable to inhibit tumor growth. In contrast, valproate combined with cisplatin and pemetrexed (C+P+V) completely abolished tumor progression ($P < 0.05$). Further evidence supporting the ability of valproate to ameliorate C+P treatment was obtained from two other models [murine AB12 (Fig. 6) and human M38K (data not shown)]. In contrast, the combined treatment was inefficient in sarcomatoid ZL34 xenografts (Fig. 6). Finally, N-acetylcysteine had only a modest or no effect on tumor growth in M38K and AB12 cells, respectively (data not shown).

These data show that valproate increases the antitumor efficiency of C+P+V in three out of four mouse xenograft models.

**Discussion**

Epidemiologic studies show that the incidence of malignant pleural mesothelioma is increasing worldwide, because of the widespread use of asbestos-containing materials over the last decades (28). Malignant pleural mesothelioma patients have a poor survival because of the usual late stage of the disease at presentation and the chemoresistance of the tumor. Although the etiology of mesothelioma is well known, therapeutic approaches have been disappointing. Currently, an effective nonsurgical therapy is still unavailable and the possibility of curative resection is extremely rare (6, 29).

In this work, we hypothesized that therapeutic efficacy was hampered by epigenetic defects and we therefore evaluated the effect of valproate on mesothelioma cells. We show that clinically relevant doses of valproate increase apoptosis induced by cisplatin and pemetrexed, the standard regimen of first-line chemotherapy in malignant pleural mesothelioma (Fig. 2). Interestingly, the combination of these three drugs is also effective in the ZL34 cell line pertaining to the sarcomatoid subtype, known to be the most chemoresistant subtype of mesothelioma (30–32). In addition, cisplatin and pemetrexed associated with valproate also triggers apoptosis in fresh biopsies isolated from malignant pleural mesothelioma patients further supporting the therapeutic significance of this regimen (data not shown). These results support other studies showing the proapoptotic effect of HDAC inhibitors (sodium butyrate and suberohydroxamic acid) used alone or in combination with chemotherapeutic agents (33, 34).

Compared with SAHA or other hydroxamic acids like trichostatin A, valproate is certainly not the most potent HDAC inhibitor (35). However, valproate has a long therapeutic history as an antiepileptic agent (36, 37). The pharmacokinetic properties (e.g. half-life of 16 to 17 hours) and bioavailability of valproate are compatible with convenient clinical treatment. The adverse effects associated with valproate treatment at therapeutic doses in humans encompassing the millimolar range are drowsiness and tremor, with thrombocytopenia being...
observed at >3 mmol/L (36, 38). In contrast, SAHA has a short half-life time (19 to 47 minutes) and yields hematologic toxicities at therapeutic concentrations (39). As an anticonvulsant and a mood stabilizer, valproate is even beneficial for the treatment of cancer-related neuropathic pain (40). In the long term, the major side effects of valproate (i.e. mainly teratogenicity and hepatotoxicity) are acceptable, at least in the context of an anticancer treatment (19). Importantly, valproate does not increase the risk of secondary cancers, as indicated by decades of epilepsy treatment (41). All these arguments favor the use of valproate compared with other HDAC inhibitors.

The onset of HDAC inhibitor–induced apoptosis parallels accumulation of acetylated histone H3 used as a general marker of cell hyperacetylation (Fig. 4). Histone hyperacetylation leads to chromatin decondensation, thereby allowing transcriptional activation of a series of genes (42, 43). An open conformation of chromatin may also favor access of cisplatin to the DNA and increase its damaging activity. Consistent with this model, valproate and DNA intercalating agents such as epirubicin or doxorubicin synergize to induce apoptosis of cancer cell lines (44, 45).

Most of currently available chemotherapeutic agents induce cell death via the intrinsic pathway requiring permeabilization of mitochondria with cytochrome c release and caspase 9 activation (46, 47). Pemetrexed induces caspases 8 and 9 cleavage but also triggers apoptosis through a caspase-independent
Valproate induces ROS production and free radical scavenging inhibits apoptosis. 

**A, ROS production.** Three mesothelioma cell lines (M14K, M38K, and ZL34) were incubated for 30 min in the presence of 5 μmol/L of H$_2$DCFDA at 37°C. Then, the cells were cultivated in a combination of valproate (2 mmol/L), SAHA (2 μmol/L), pemetrexed (P; 200 μmol/L), and cisplatin (C; 10 μmol/L) alone or in combination. After 24 h of culture, ROS were detected by flow cytometry and are presented as the mean fluorescence intensities of chloromethyldichlorofluorescein. As control, cells were cultivated in presence of 100 μmol/L of H$_2$O$_2$. ROS were scavenged with 10 mmol/L of N-acetylcysteine (NAC). The results are presented as means and SD of three independent experiments. *, **, and *** denote the statistically significant differences according to the paired Student’s t test $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

**B, apoptosis.** Three mesothelioma cell lines (M14K, M38K, and ZL34) were cultivated for 24 h in the presence of a combination of valproate (V; 2 mmol/L), SAHA (S; 2 μmol/L), pemetrexed (P; 200 μmol/L), and cisplatin (C; 10 μmol/L). Half of the samples were pretreated with 10 mmol/L of N-acetylcysteine to inhibit ROS production (NAC). Apoptosis was evaluated by flow cytometry after Annexin V-7AAD labeling. An-positive/7AAD-negative and An-positive/7AAD-positive cells were considered as early and late apoptotic, respectively. The results are represented as the means and SD of three independent experiments. *, ** denote the statistically significant differences according to the paired Student’s t test $P < 0.05$ and $P < 0.01$, respectively.
pathway that includes release of apoptosis-inducing factor (48). SAHA induces apoptosis in human colon adenocarcinoma cells by dissipation of mitochondrial transmembrane potential and activation of executioner caspases. Moreover, SAHA up-regulates the death receptor DR5, activating caspase-8 and down-regulating FLICE inhibitory protein and Akt, two proteins that exert an inhibitory role in apoptosis (49). The mechanism by which the combination of cisplatin and pemetrexed promotes apoptosis is clearly dependent on caspase 8 and also requires caspase 9 activity (Fig. 3). Inclusion of HDAC inhibitors in the cisplatin and pemetrexed regimen does not modify this caspase dependency profile, suggesting that increased apoptosis does not directly result from coordinate activation of two independent pathways. In fact, the extrinsic and intrinsic pathways can interact via cleavage of Bid by caspase 8 and its translocation to the mitochondria (50, 51). The cleaved carboxyterminal portion of Bid stimulates release of cytochrome c from mitochondria and loss of membrane potential (52). Importantly, Bid protein processing in truncated t-Bid and cytochrome c release from mitochondria are significantly increased in the presence of valproate and SAHA (Fig. 4), providing a mechanistic rationale for improvement of the proapoptotic efficacy of cisplatin and pemetrexed. This model is also consistent with the ability of HDAC inhibitors to promote tumor-selective apoptosis through activation of the death receptor pathway (53, 54). In fact t-Bid is a key mediator of ROS production following death receptor activation and FLICE inhibitory protein degradation (55).

Interestingly, the mechanism of valproate-induced apoptosis also involves production of ROS, further extending observations in other systems (56, 57). Experimental evidence includes the specific stimulation of ROS by valproate but interestingly not by SAHA (Fig. 5). In M14K and M38K cells, efficient inhibition of ROS with N-acetylcysteine correlates with reduced apoptosis, suggesting a potential link between both processes. Conversely, when ROS production could not be abrogated as observed in sarcomatoid ZL34 cell line, the apoptotic rates were unaltered by N-acetylcysteine addition. However, apoptosis induced by the combination of cisplatin and pemetrexed could be inhibited by N-acetylcysteine without significant modification of ROS production. Moreover, valproate stimulated ROS without altering apoptotic rates. All these observations are consistent with a threshold level of ROS production that allows cell survival and above which apoptosis occurs. For example, ROS production is exacerbated when valproate is combined with cisplatin and pemetrexed. In fact, cisplatin forms highly reactive complexes which bind to nucleophilic groups such as GC-rich sites in DNA, inducing intrastrand and interstrand cross-links. Cisplatin damages mitochondrial DNA, which results in electron transport chain dysfunction and subsequent enhanced ROS generation (46, 58). Cisplatin further inhibits glutathione peroxidase and superoxide dismutase, thereby reducing cellular capacity to metabolize free oxygen radicals produced normally by the mitochondria (59). Together, our data indicate that the mechanism by which valproate combined to cisplatin and pemetrexed triggers maximal levels of apoptosis includes caspase activation, Bid processing, ROS production, and cytochrome c release.

In the absence of an efficient treatment for mesothelioma, our report indicates that the inclusion of HDAC inhibitors to the association of cisplatin and pemetrexed, the standard regimen of first-line chemotherapy for malignant pleural mesothelioma, may improve the response to treatment. Preclinical models of engrafted mice further support this novel and promising therapeutic option (Fig. 6). Based on these results, the potential clinical benefit of addition of valproate to a classical chemotherapy used in malignant pleural mesothelioma patients is being presently evaluated in a phase II clinical trial in refractory or recurrent malignant pleural mesothelioma (protocol 01062 at http://www.elcwp.org/). Independently of

Fig. 6. Evaluation of mesothelioma tumor growth in mice treated by a combination of chemotherapeutic agents. Two human mesothelioma cell lines (M14K and ZL34) were injected s.c. in SCID mice in the presence of 50% of matrigel. The murine AB12 cell line was injected s.c. in BALB/c mice. After tumor development, i.p. injections of valproate (400 mg/kg/d), SAHA (50 mg/kg/d), cisplatin (C; a single dose of 2 mg/kg), and pemetrexed (P; 30 mg/kg/d) were done, as indicated. The tumor volume (in mm$^{3}$) was calculated at regular intervals of time (in days). Groups of at least 6 mice were tested in each experimental condition. * denotes the statistical significant difference according to the paired Student’s $t$ test $P < 0.05.$
the outcome of this trial, the present study supports the argument for a potential activity of valproate when added to chemotherapy combining cisplatin and pemetrexed with the ultimate goal of improving the clinical outcome of patients with malignant pleural mesothelioma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Valproate, in Combination with Pemetrexed and Cisplatin, Provides Additional Efficacy to the Treatment of Malignant Mesothelioma

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