



A 5-aza-2'-deoxycytidine/valproate combination induces cytotoxic T-cell response against mesothelioma

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ABSTRACT: Malignant pleural mesothelioma (MPM) is an aggressive tumour with a limited response to conventional therapy. The aim of this study was to evaluate the anticancer effect of a DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-azaCdR), and two histone deacetylase inhibitors, valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA).

Human mesothelioma cells were treated with each epigenetic drug, either alone or in combinations. The cytotoxic effects on treated cells and the expression of specific tumour antigens were evaluated. The recognition of treated cells by a specific CD8⁺ T-cell clone was also measured. Additionally, the effect of combined treatments was tested in a murine model of mesothelioma.

We showed that VPA and SAHA synergised with 5-azaCdR to kill MPM cells and induce tumour antigen expression in the remaining living tumour cells. As a consequence, tumour cells expressing these antigens were recognised and lysed by specific CD8⁺ cytotoxic T-cells. *In vivo*, treatment with 5-azaCdR/VPA inhibited tumour growth, and promoted lymphocyte infiltration and an immune response against tumour cells.

Appropriate epigenetic drug combinations, in addition to inducing mesothelioma cell death, also affect the immunogenic status of these cells. This property could be exploited in clinical investigations to develop MPM treatments combining chemotherapeutic and immunotherapeutic approaches.

KEYWORDS: 5-aza-2'-deoxycytidine, immunotherapy, mesothelioma, suberoylanilide hydroxamic acid, tumour antigen, valproic acid

Malignant pleural mesothelioma (MPM) is an aggressive tumour of the pleura that is usually associated with chronic asbestos exposure [1]. Worldwide, the incidence of MPM is increasing and is expected to peak around the year 2020 [2]. MPM treatments include chemotherapy, radiotherapy and surgery, but are of limited efficacy, necessitating the development of new therapeutic strategies. Numerous pre-clinical and clinical studies have proved that hypomethylating drugs, such as 5-aza-2'-deoxycytidine (5-azaCdR; decitabine), and histone deacetylase (HDAC) inhibitors, such as valproic acid (VPA; depakine) and suberoylanilide hydroxamic acid (SAHA; vorinostat), have potent anticancer activity and promising therapeutic potential [3–11].

The DNA methyltransferase (DNMT) inhibitor decitabine allows the expression of silenced genes through the demethylation of CpG islands. This drug has recently demonstrated clinical efficacy in the treatment of haematopoietic malignancies [12].

The use of HDAC inhibitors aims to block chromatin compaction due to histone deacetylation, thereby increasing gene expression [13]. Among HDAC inhibitors, depakine and vorinostat are currently being evaluated, alone and in combination, as cytotoxic agents for MPM treatment [8, 9, 14]. In these clinical studies of MPM, and for many others cancer types, decitabine and HDAC inhibitors are being evaluated for their ability to slow down tumour progression by inducing cell cycle arrest, differentiation and/or apoptosis.

In addition, these drugs may also have an impact on the immune response. As spontaneous regression has been observed in mesothelioma patients with infiltration of tumour-specific lymphocytes [15], immunotherapy strategies for the treatment of MPM should be considered [16]. However, the candidate antigens that can be targeted in MPM are poorly defined. One way to bypass this lack of identified antigens for MPM is to force tumour cells to express tumour-associated antigens

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(TAAs). With the aim of favouring TAA expression, it has been shown that hypomethylating drugs and/or HDAC inhibitors, in addition to restoring a normal epigenetic status in cancer cells, can also induce tumour antigen expression [17–21].

The aim of our investigation was to demonstrate the combined effect of the epigenetic drugs, DNMT and HDAC inhibitors, on both cell toxicity and new expression of TAAs that could activate a cytotoxic T-cell response. Thus, we investigated the toxicity of 5-azaCdR, VPA and SAHA, alone and in sequential combinations, on human MPM cell lines. The effects of these drugs on the expression of several cancer testis antigens (CTAs) (such as NY-ESO-1 (New York oesophageal cancer), and MAGE-A1 and A3 (melanoma-associated antigens)), which are of increasing interest as immunotherapeutic targets, were also analysed, as well as recognition of treated cells by cytotoxic T-cells. Our study showed that VPA and SAHA trigger MPM cell death, and synergise with 5-azaCdR to induce NY-ESO-1 expression in the remaining living cells, which became sensitive to lysis mediated by NY-ESO-1-specific cytotoxic T-cells. Additionally, using a mouse model of mesothelioma, we report *in vivo* tumour regression associated with lymphocyte infiltrates after sequential 5-azaCdR and VPA treatment. These infiltrates contained CD8a+ lymphocytes able to secrete interferon (IFN)- γ in response to tumour cells.

MATERIALS AND METHODS

Cell culture

Human epithelioid mesothelioma cell lines were established from pleural effusions in our laboratory [22]. The human melanoma cell line M117 was provided by N. Labarrière (INSERM, U892, Nantes, France). Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU·mL⁻¹ penicillin and 0.1 mg·mL⁻¹ streptomycin (complete medium). Human mesothelial cells were isolated from normal pleura by enzymatic disaggregation, and cultured in complete medium supplemented with hydrocortisone (0.4 μ g·mL⁻¹), epidermal growth factor (10 ng·mL⁻¹), insulin (0.5 μ g·mL⁻¹) and nonessential amino acids. 5-azaCdR, VPA and cell culture reagents were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). SAHA was provided by P. Bertrand (CNRS UMR 6514, University of Poitiers, Poitiers, France). After drug treatments, cell viability was assessed by TO-PRO-3 iodide labelling (Invitrogen, Cergy-Pontoise, France) and flow cytometry analysis.

The human leukocyte antigen (HLA)-A*0201-restricted, NY-ESO-1(157–165)-specific CD8+ T-cell clone M117.32H was obtained from tumour-infiltrating lymphocytes of melanoma patient 117 and cultured as described by FONTENEAU *et al.* [23].

RNA extraction and real-time RT-PCR

Total RNA was extracted with the RNeasy Mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. 1 μ g total RNA was reverse-transcribed using Moloney murine leukaemia virus reverse transcriptase (Invitrogen). PCR reactions were carried out using 10 \times QuantiTect Primer Assays (Qiagen) and RT² Real-Time SYBR-Green/ROX PCR master mix (Tebu-bio, Le-Perray-en-Yvelines, France), according to the manufacturers' instructions.

Activation of HLA-A*0201-restricted, NY-ESO-1-specific CD8+ T-cells

Tumour cells were co-cultured with NY-ESO-1-specific CD8+ T-cells in complete medium containing 10 μ g·mL⁻¹ brefeldin A (Sigma-Aldrich) for 5 h at 37°C and washed. Cells were stained with allophycocyanin-conjugated mouse anti-human CD8 and phycoerythrin (PE)-conjugated mouse anti-human IFN- γ monoclonal antibodies (BD Biosciences, Le Pont de Claix, France) using methods described by SIGALOTTI *et al.* [19]. CD8 and IFN- γ expression were analysed by flow cytometry.

Cytotoxicity assay

Tumour cells were incubated with Na₂⁵¹CrO₄ (PerkinElmer, Courtaboeuf, France) for 1 h at 37°C and either pulsed or not with 10 μ M NY-ESO-1(157–165) peptide for 1 h at 4°C. Cells were subsequently co-cultured with HLA-A*0201-restricted NY-ESO-1-specific CD8+ T-cells for 4 h at 37°C, in triplicate. Each supernatant was collected and added to a scintillation liquid cocktail (OptiPhase Supermix; PerkinElmer) before liquid scintillation counting. The percentage of specific lysis was calculated as 100 \times (experimental release - spontaneous release)/(maximum release - spontaneous release). The spontaneous release of ⁵¹Cr was determined from target T-cells cultured alone. The maximum release of ⁵¹Cr was obtained from target T-cells lysed in medium containing 1% Triton X-100.

Animal experiments

The experiments reported here were carried out in compliance with the guidelines of the European Union for the care and use of animals in research protocols. 5 \times 10⁶ AK7 murine mesothelioma cells per mouse [24] were administered intraperitoneally to four groups of C57BL/6 mice (Elevage Janvier, Le Genest-St-Isle, France) (day 0). Group 1 did not receive any further treatment. Groups 2 and 4 were given three successive *ip.* injections of 5-azaCdR (4 mg·kg⁻¹) at days 7, 9 and 14. VPA (5 mM) was added to the drinking water of mice in groups 3 and 4 from day 10 to day 20. All animals were necropsied on day 22.

Histopathology and immunohistochemistry

Tumours and tissues were fixed in 4% formaldehyde in PBS, embedded in paraffin, cut into 5- μ m sections and stained with haematoxylin–eosin–safran.

Immunohistochemistry was performed on tumour slices (paraffin-embedded) using standard techniques. The primary antibody, anti-Foxp3 (Abcam, Paris, France), was used at a dilution of 1/100. N-Histofine Simple Stain Mouse MAX Peroxidase (Nichirei Biosciences, Tokyo, Japan) was used as the detection reagent.

IFN- γ ELISPOT assay

Spleens, as well as AK7 tumours, were pooled for each group of mice (control and 5-azaCdR/VPA) and were dissociated using the gentleMACS Dissociator (Miltenyi Biotech, Paris, France), according to the manufacturer's optimised protocols. Cell suspensions were 70- μ m-filtered, aseptically, and red blood cells were lysed with BD Pharm Lyse solution (BD Biosciences). Living cells were labelled with anti-CD8a (Ly-2) MicroBeads (Miltenyi Biotech). CD8a+ cells were positively sorted using MACS columns and a MACS separator (Miltenyi Biotech). CD8a+ splenocytes (5 \times 10⁵ cells per well) and CD8a+ tumour-infiltrating lymphocytes (1 \times 10⁵ cells per well) were

cultured in complete medium supplemented with 50 μM 2-mercaptoethanol (Merck, Nottingham, UK) at 37°C in a 96-well ELISPOT plate for 36 h. The stimulator cells were AK7 cells (5×10^4 cells per well), pre-treated or not with 0.5 μM 5-azaCdR (72 h) and 5 mM VPA (48 h). The ELISPOT assay was performed in triplicate according to the manufacturer's instructions (BD Biosciences). Spots were counted automatically using an ELISPOT plate reader (Autoimmun Diagnostika GmbH, Strassberg, Germany).

Statistical analysis

Statistical significance was calculated using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Comparisons between groups were made using the Mann–Whitney U-test or Kruskal–Wallis ANOVA followed by Dunn's post-test (multiple comparisons). A two-tailed p-value <0.05 was considered as significant.

RESULTS

Toxic and proliferative effects of 5-azaCdR, VPA and SAHA on MPM cells

Carcinogenesis results from a combination of genetic abnormalities and epigenetic modifications leading to dysregulation of genes involved in cell cycle control and apoptosis. Modifications of chromatin depend on the activities of enzymes such as histone acetyltransferases (HATs), HDACs and DNMTs, which are considered to be good targets for cancer therapy. Thus, we first studied the toxic effects of the HDAC inhibitors VPA and

SAHA, and the hypomethylating drug 5-azaCdR on MPM tumour cells, using TO-PRO-3 iodide staining and flow cytometry. These drugs were used at concentrations known to be compatible with their biological activity. After 48 h, VPA or SAHA triggered MPM tumour cell death with a median inhibitory concentration (IC₅₀) of 10 mM and 10 μM , respectively (fig. 1). This toxic effect was linked to increased MPM cell apoptosis, as indicated by annexin V staining (data not shown). Conversely, 5-azaCdR showed no toxicity and no obvious effect on MPM cell proliferation.

Effects of 5-azaCdR, VPA and SAHA on TAA expression

MPM displays multiple epigenetic abnormalities that lead to the silencing of tumour suppressor genes [25]. Such observations suggest that MPM may be a good target for epigenetic drugs that would favour the expression of new, tumour-associated genes. Thus, we studied the impact of 5-azaCdR, VPA and SAHA on the expression of three TAAs (NY-ESO-1, MAGE-A1 and MAGE-A3). Using quantitative RT-PCR (RT-qPCR), we showed that NY-ESO-1 mRNA was undetectable in all untreated MPM cell lines, and that MAGE-A1 and -A3 mRNAs were also undetectable in half of the tested cell lines. After treatment, 5-azaCdR induced or upregulated the expression of the three antigens tested in a dose-dependent manner (fig. 2). A concentration of 0.5 μM 5-azaCdR was sufficient to achieve optimal induction levels. In contrast, VPA or SAHA alone had no effect on mRNA expression of the TAA tested (data not shown).

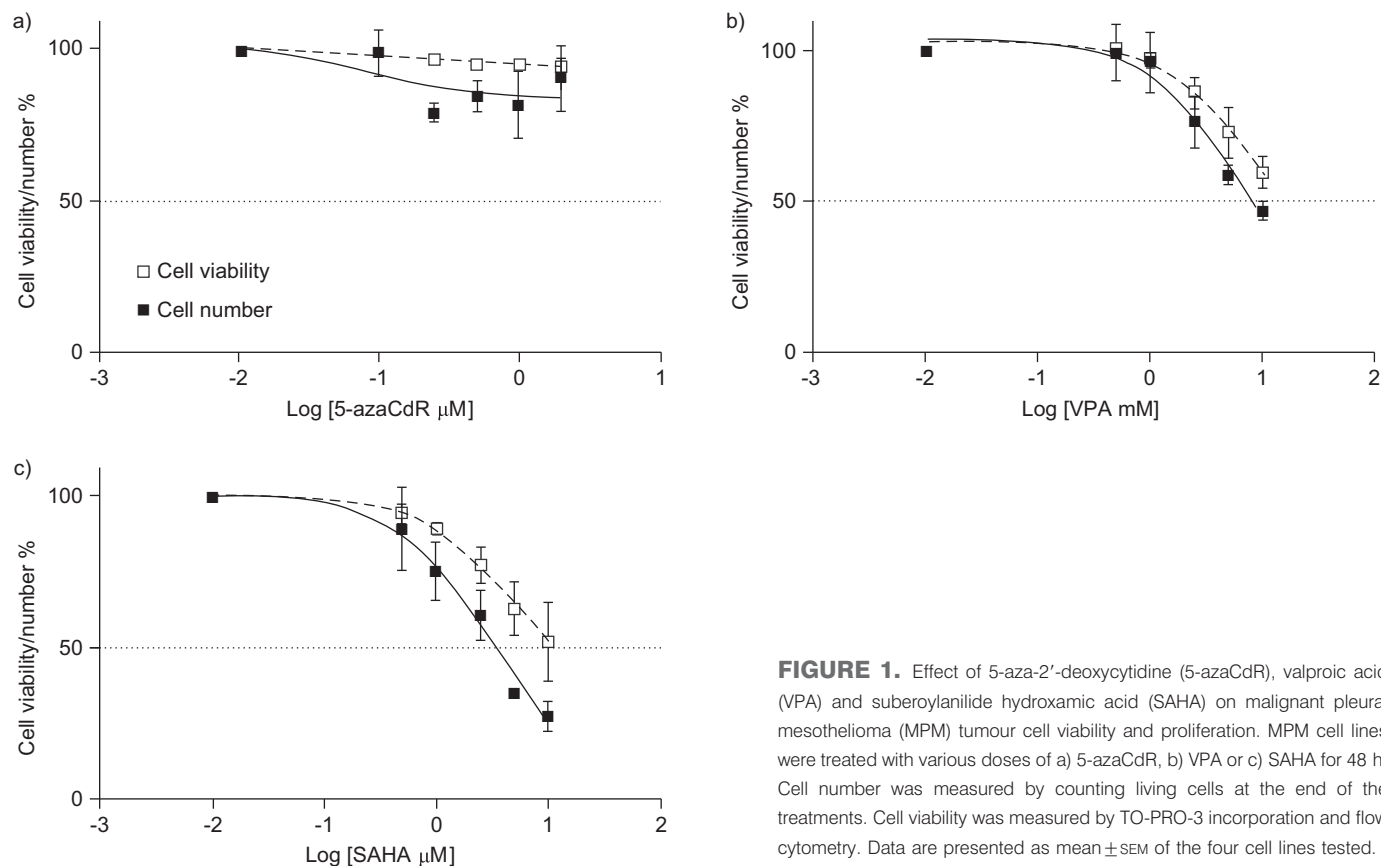


FIGURE 1. Effect of 5-aza-2'-deoxycytidine (5-azaCdR), valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA) on malignant pleural mesothelioma (MPM) tumour cell viability and proliferation. MPM cell lines were treated with various doses of a) 5-azaCdR, b) VPA or c) SAHA for 48 h. Cell number was measured by counting living cells at the end of the treatments. Cell viability was measured by TO-PRO-3 incorporation and flow cytometry. Data are presented as mean \pm SEM of the four cell lines tested.

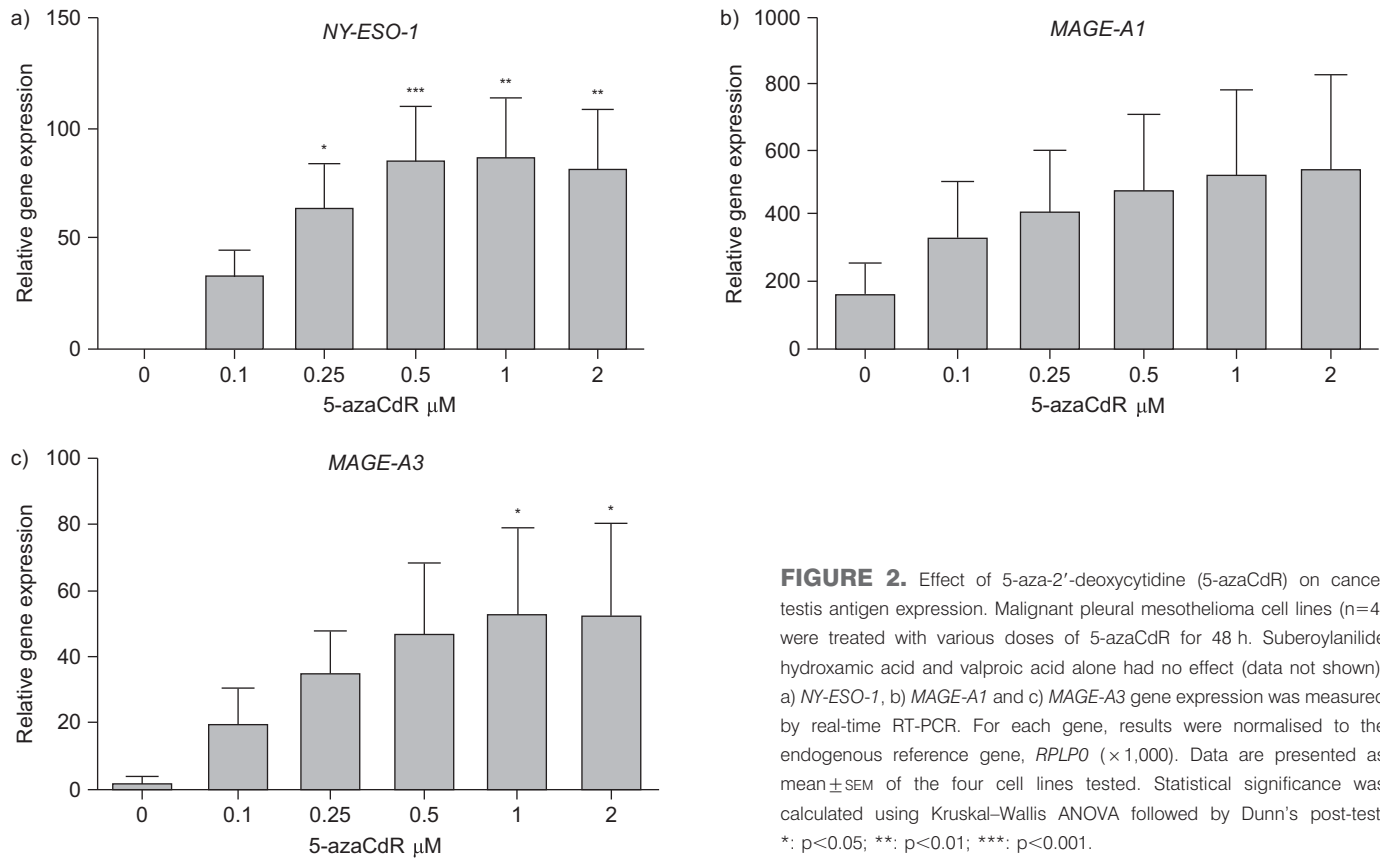


FIGURE 2. Effect of 5-aza-2'-deoxycytidine (5-azaCdR) on cancer testis antigen expression. Malignant pleural mesothelioma cell lines (n=4) were treated with various doses of 5-azaCdR for 48 h. Suberoylanilide hydroxamic acid and valproic acid alone had no effect (data not shown). a) *NY-ESO-1*, b) *MAGE-A1* and c) *MAGE-A3* gene expression was measured by real-time RT-PCR. For each gene, results were normalised to the endogenous reference gene, *RPLP0* ($\times 1,000$). Data are presented as mean \pm SEM of the four cell lines tested. Statistical significance was calculated using Kruskal-Wallis ANOVA followed by Dunn's post-test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

VPA and SAHA enhance the expression of TAAs in MPM tumour cells pre-treated with 5-azaCdR

Many studies have evaluated the effects of combined treatment of cancer cells with a hypomethylating agent (especially 5-azaCdR) and a HDAC inhibitor [26, 27]. Since these drug types affect two cooperative epigenetic mechanisms, we sought to determine the best drug combination schedules. Thus, in order to improve treatment efficiency, we first assessed whether VPA treatment before or after 5-azaCdR treatment would be more efficient. Two different schedules of treatment with 5-azaCdR and VPA were tested. MPM cell lines were treated with 0.5 μ M 5-azaCdR followed by 5 mM VPA, or with 5 mM VPA followed by 0.5 μ M 5-azaCdR (fig. 3a). Analysis of *NY-ESO-1* mRNA expression by RT-qPCR showed an induction after both of these sequential treatments. However, treatment with 5-azaCdR/VPA, as compared with VPA/5-azaCdR, was associated with much higher *NY-ESO-1* mRNA expression levels in all MPM cell lines tested. The optimal schedule of sequential 5-azaCdR/VPA treatment was investigated (fig. 3b). The best inductions of *NY-ESO-1* expression were observed after sequential treatments with 0.5 μ M 5-azaCdR (48 h)/5 mM VPA (48 h) and 0.5 μ M 5-azaCdR (72 h)/5 mM VPA (48 h).

Genetic abnormalities and epigenetic modifications are characteristics of chromatin alteration that lead to carcinogenesis. Thus, epigenetic drug inhibitors should not have an effect on normal cells. The effects of the most effective combination, 5-azaCdR/VPA, on *NY-ESO-1* expression in normal mesothelial cells (NMCs) were evaluated. NMC primary cultures were obtained from pleural biopsies of patients undergoing cardiac

surgery. NMC cultures were positive for the normal mesothelium markers calretinin and cytokeratins-5 and -6 (data not shown). *NY-ESO-1* mRNA was not detected in NMCs, either before or after treatment with 5-azaCdR/VPA (fig. 3c). Conversely, *NY-ESO-1* was induced in MPM-treated cells, but to a lower level than that observed in the melanoma cell line M117. Similar results were obtained for *MAGE-A1* and *-A3* (data not shown).

In another set of experiments, we compared the effects of the combinations 5-azaCdR/VPA and 5-azaCdR/SAHA on TAA expression and cell proliferation. MPM cells were treated with 0.5 μ M 5-azaCdR and with either 5 mM VPA or 2.5 μ M SAHA (fig. 4). No effect of 5-azaCdR on cell proliferation was observed after treatment. Conversely, the combination of 5-azaCdR with VPA or with SAHA (used at doses below their IC₅₀) led to a 50% inhibition of cell proliferation (40% when the HDAC inhibitor was used alone). However, as previously noted with HDAC inhibitor alone, no complete toxicity was observed with combination treatments. Furthermore, 5-azaCdR/VPA treatment was associated with higher TAA mRNA levels than treatment with 5-azaCdR alone or the 5-azaCdR/SAHA combination. Thus, we focused on the combination 5-azaCdR/VPA for subsequent experiments.

HLA-A*0201-restricted, NY-ESO-1-specific CD8⁺ T-cells respond to all HLA-A*0201+, 5-azaCdR/VPA-treated MPM cell lines

NY-ESO-1 is considered a very valuable target for T-cell-based immunotherapy [28]. Thus, we evaluated whether the *NY-ESO-1*

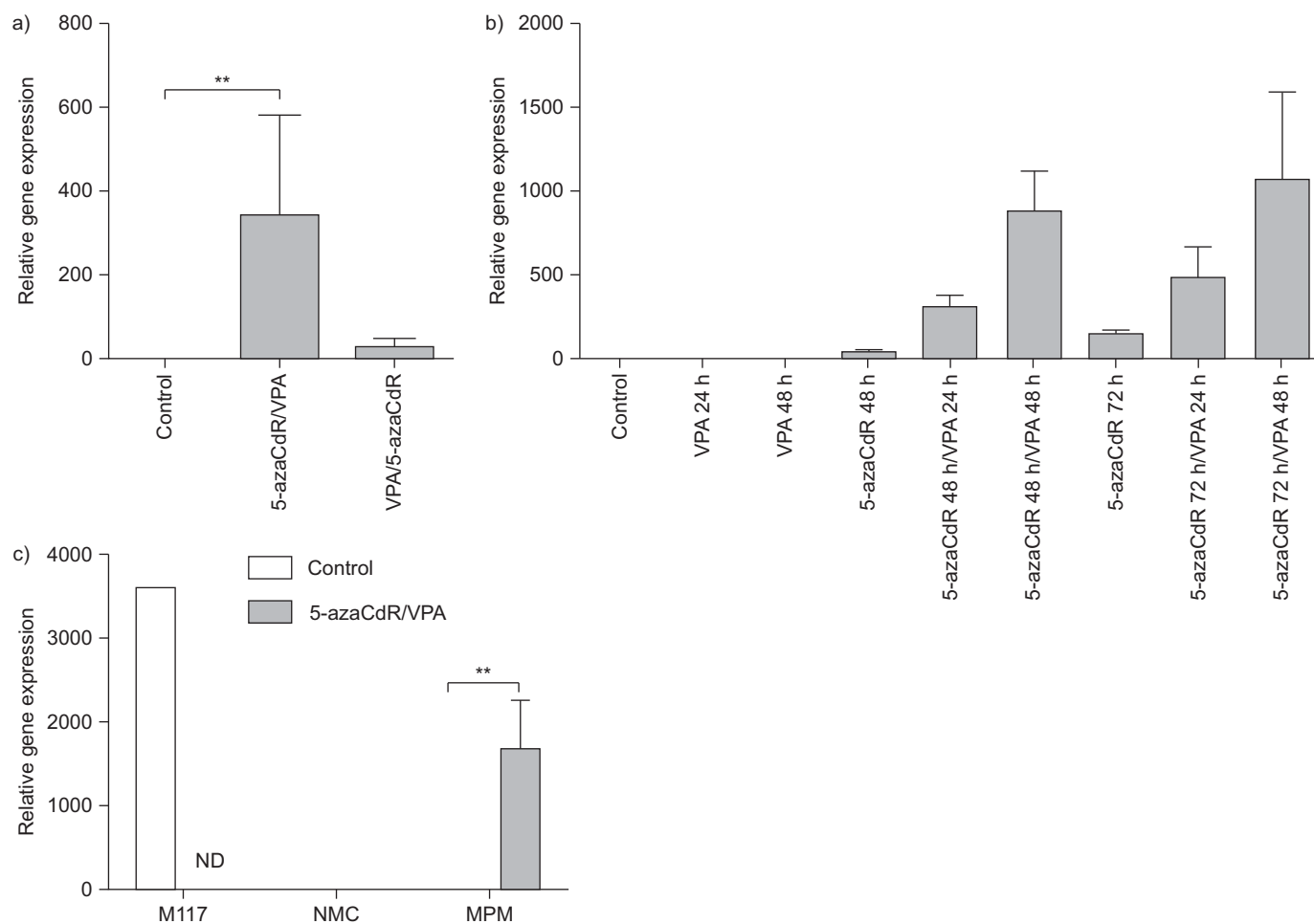


FIGURE 3. Impact of different schedules of 5-aza-2'-deoxycytidine (5-azaCdR) and valproic acid (VPA) administration on *NY-ESO-1* expression in malignant pleural mesothelioma (MPM) cells and normal mesothelial cells (NMCs). a) *NY-ESO-1* expression was assessed by quantitative RT-PCR (RT-qPCR) in different MPM cell lines (n=5) treated sequentially with 0.5 μ M 5-azaCdR for 48 h and 5 mM VPA for 24 h (5-azaCdR/VPA), or with 5 mM VPA for 24 h and 0.5 μ M 5-azaCdR for the next 48 h (VPA/5-azaCdR). b) *NY-ESO-1* expression was assessed by RT-qPCR in two MPM cell lines treated with 0.5 μ M 5-azaCdR and 5 mM VPA, alone or in combination, with different time schedules. c) NMCs in primary cultures (n=2) were treated sequentially with 0.5 μ M 5-azaCdR for 72 h and 5 mM VPA for 48 h. *NY-ESO-1* expression in NMCs was compared to that obtained in five MPM cell lines, and the melanoma cell line M117 as a positive control. *NY-ESO-1* expression was normalised to the endogenous reference gene, *RPLP0* ($\times 1,000$). Data are presented as mean \pm SEM. Statistical significance was calculated a) using Kruskal–Wallis ANOVA followed by Dunn's post-test or c) Mann–Whitney U-test. ND: not determined. **: $p < 0.01$.

expression observed in 5-azaCdR/VPA-treated, HLA-A*0201+ MPM cells was able to promote their recognition by an NY-ESO-1(157–165)/HLA-A*0201-specific CD8+ T-cell clone. This T-cell clone, M117.32H, was obtained from limiting-dilution cultures of tumour-infiltrating lymphocytes from a melanoma patient and recognised the autologous tumour cell line M117 (N. Labarrière; personal communication). Furthermore, this clone exhibited 100% staining with PE-conjugated NY-ESO-1(157–165)/HLA-A*0201 and no staining with HLA-A*0201 tetramer loaded with an irrelevant peptide (data not shown).

In the first set of experiments, IFN- γ production by the CD8+ T-cell clone, in response to an MPM cell line treated, or not, with 5-azaCdR/VPA, was tested (fig. 5a). Untreated and treated tumour cells were washed and then co-cultured with the NY-ESO-1-specific T-cell clone. No T-cell clone response was observed with tumour cells that were not treated with 5-azaCdR/VPA. IFN- γ production by the clone was observed by

intracytoplasmic staining only in response to 5-azaCdR/VPA-treated cells. Under some conditions, IFN- γ was added with VPA to the culture medium, in order to increase the expression of HLA class I molecules at the surface of MPM cells (data not shown) and, therefore, their recognition by CD8+ T-cells. As expected, a slightly better activation of the clone was observed (e.g. $\leq 32.3\%$ of cells *versus* 26.2% for the MPM cell line meso96) when tumour cells were pre-treated with exogenous IFN- γ .

The level of activation of the clone in response to 5-azaCdR/VPA-treated MPM cell lines and its level of recognition of the control autologous M117 melanoma cell line were then compared. All 5-azaCdR/VPA-treated, HLA-A2+ MPM cell lines were recognised by the clone, but at levels lower than the level of recognition of M117 (fig. 5b). As a control, it was also demonstrated that the HLA-A*0201- MPM cell line was not recognised, as it did not express the NY-ESO-1(157–165)-presenting

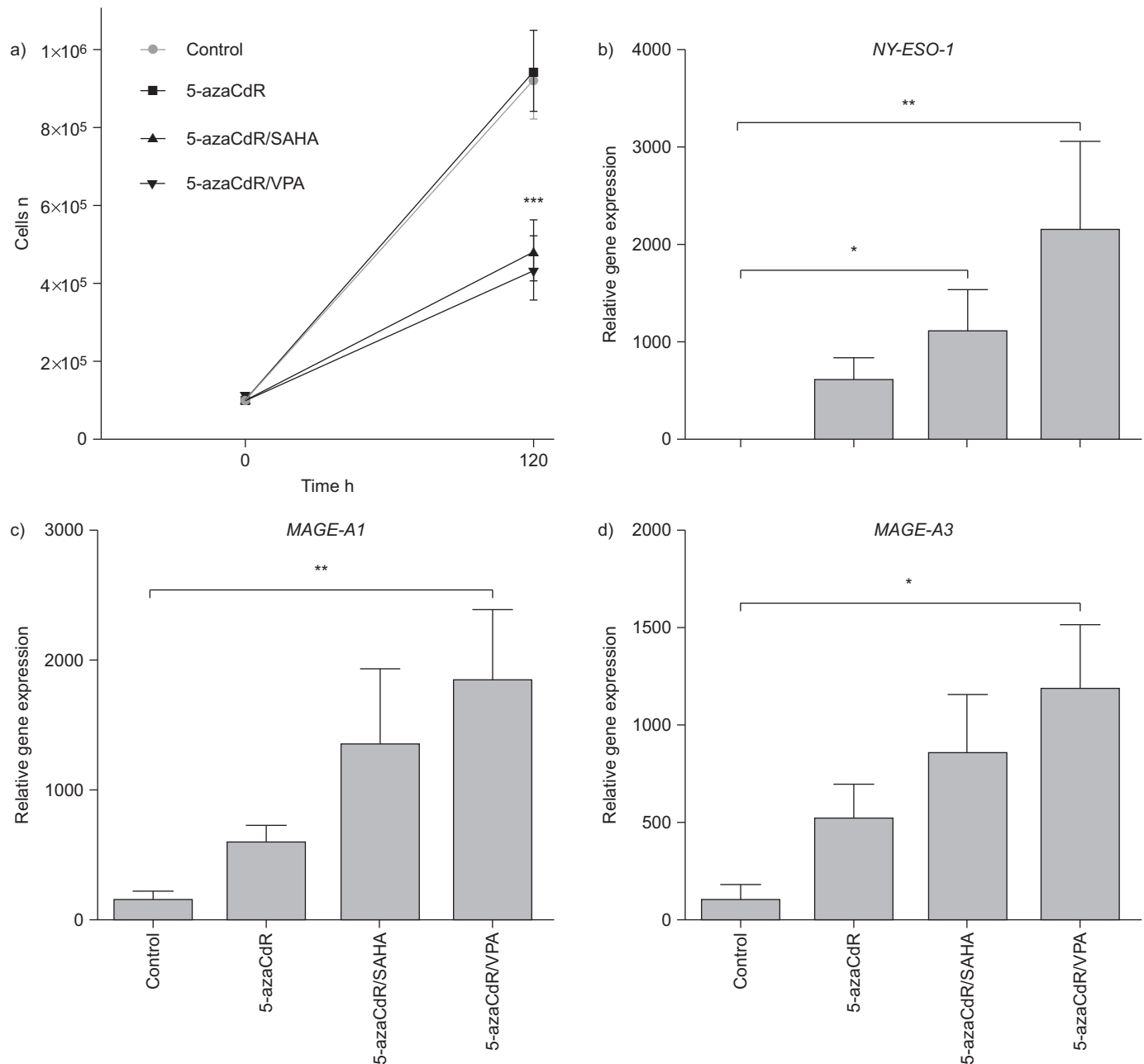


FIGURE 4. Effects of the combinations 5-aza-2'-deoxycytidine (5-azaCdR)/valproic acid (VPA) and 5-azaCdR/suberoylanilide hydroxamic acid (SAHA) on cell proliferation and cancer testis antigen expression. Malignant pleural mesothelioma cell lines were treated sequentially with 0.5 μ M 5-azaCdR for 72 h and 5 mM VPA or 2.5 μ M SAHA for 48 h. a) The numbers of living cells were measured at the end of the treatments. Each curve represents the mean \pm SEM growth of the six cell lines tested. b) *NY-ESO-1*, c) *MAGE-A1* and d) *MAGE-A3* gene expression were measured by real-time RT-PCR. For each gene, results were normalised to the endogenous reference gene, *RPLP0* ($\times 1,000$). Data are presented as mean \pm SEM. Statistical significance was calculated using a) two-way ANOVA, or b–d) Kruskal–Wallis ANOVA followed by Dunn's post-test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

HLA-A*0201 molecule. Interestingly, T-cell IFN- γ production in response to all treated HLA-A*0201+ cell lines confirmed that 5-azaCdR/VPA-treated cell lines express enough NY-ESO-1 protein to efficiently process and present the peptide on their surface and to induce their recognition by an NY-ESO-1-specific CD8+ T-cell clone. Finally, all the clone responses were partially inhibited (mean \pm SEM 56.6 \pm 7.7%) by the presence of a blocking anti-HLA-A*0201 mAb (clone BB7.2) in the T-cell clone/MPM cell line co-cultures.

In addition, we tested whether T-cell IFN- γ production in response to 0.5 μ M 5-azaCdR (72 h)/5 mM VPA (48 h)-treated HLA-A*0201+ MPM tumour cells was accompanied by tumour cell lysis by the NY-ESO-1-specific CD8+ T-cells. Cytotoxicity was undetectable in untreated MPM cells, as assessed by ⁵¹Cr release (fig. 5c). As expected, MPM cells were lysed when they were pre-treated with 10 μ M NY-ESO-1(157–165) peptide or when they were treated with 5-azaCdR/VPA. Overall, the clone's cytotoxic activity was well correlated with its IFN- γ

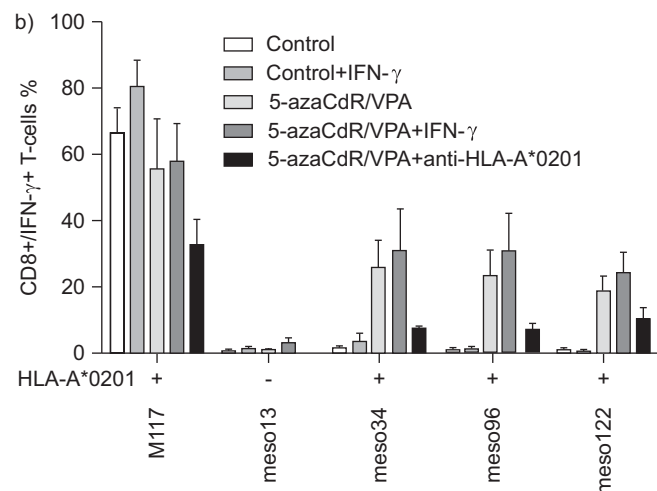
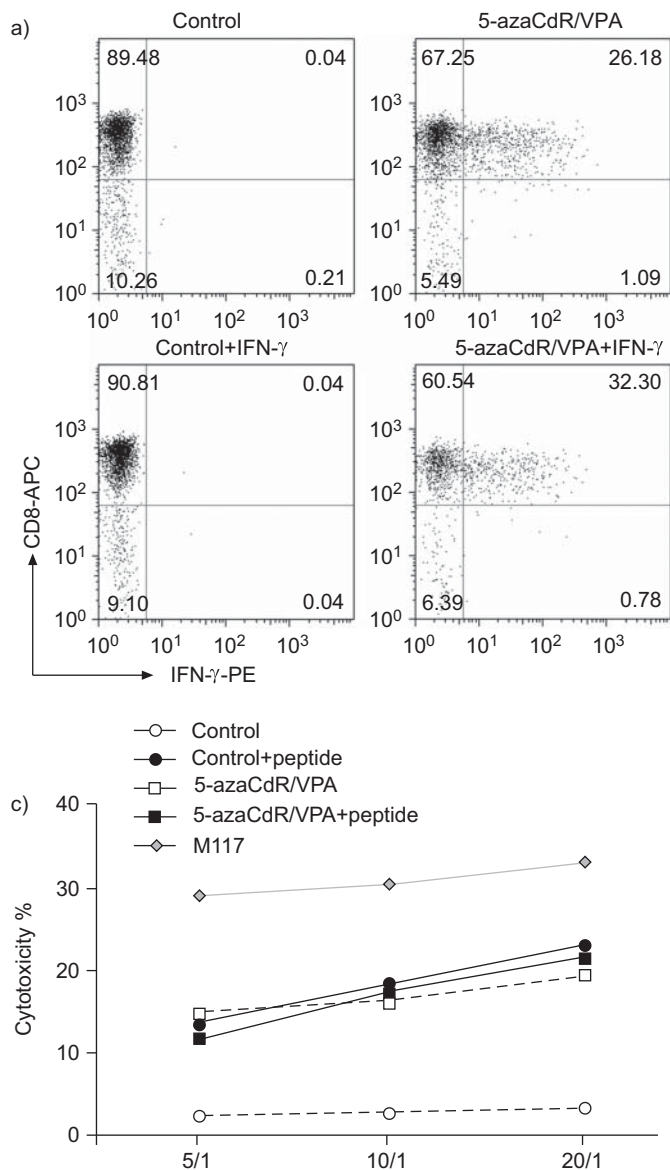


FIGURE 5. Recognition of 5-aza-2'-deoxycytidine (5-azaCdR)/valproic acid (VPA)-treated, human leukocyte antigen (HLA)-A*0201+ malignant pleural mesothelioma (MPM) cell lines by a HLA-A*0201/NY-ESO-1(157–165)-specific CD8+ T-cell clone. a) Interferon (IFN)- γ production by the NY-ESO-1-specific CD8+ T-cell clone in response to meso96 treated or not with 0.5 μ M 5-azaCdR for 72 h and 5 mM VPA for 48 h with or without 500 IU·mL⁻¹ IFN- γ for 48 h. IFN- γ production was measured by intracytoplasmic staining of IFN- γ and surface staining of CD8, followed by flow cytometry analysis. b) IFN- γ production by the NY-ESO-1-specific CD8+ T-cell clone in response to the autologous melanoma cell line (M117), a HLA-A*0201-MPM cell line (meso13) and three HLA-A*0201+ MPM cell lines (meso34, meso96 and meso122), treated or not with 0.5 μ M 5-azaCdR (72 h)/5 mM VPA (48 h), with or without IFN- γ (48 h) and a blocking anti-HLA-A*0201 monoclonal antibody. c) Cytotoxic activity of the NY-ESO-1-specific CD8+ T-cell clone in response to the autologous melanoma cell line (M117) and an HLA-A*0201+ MPM cell line (meso96) treated or not (control) with 0.5 μ M 5-azaCdR (72 h)/5 mM VPA (48 h) and pulsed or not with 10 μ M NY-ESO-1(157–165) peptide. Effector (HLA-A*0201-restricted, NY-ESO-1-specific CD8+ T-cells) to target (tumour cells) ratios of 5/1, 10/1 and 20/1 were used. Cytotoxic activity was measured by ⁵¹Cr release. Experiments were performed in triplicate for each data point. APC: allophycocyanin; PE: phycoerythrin.

production, suggesting that 5-azaCdR/VPA treatment of MPM tumour cells induces sufficient NY-ESO-1 expression to cause substantial recognition and lysis of tumour cells by NY-ESO-1-specific CD8+ T-cells.

VPA, in conjunction with 5-azaCdR, inhibits tumour growth in a murine model of mesothelioma

In order to assess the *in vivo* efficiency of the combination of 5-azaCdR and VPA, we used C57BL/6 mice engrafted with AK7 cells. In pilot studies, we showed that these cells, similarly to human MPM cells, expressed TAAs equivalent to human CTAs, such as the tumour rejection antigen P1A, upon 5-azaCdR/VPA treatment (data not shown). We also determined that *i.p.* injection of 5×10^6 AK7 cells represented the best tumorigenic dose. The administration schedule of 5-azaCdR was based on those described by Guo *et al.* [29], who found that *i.p.* injections of 5-azaCdR (1 mg·kg⁻¹ body weight) twice daily for five consecutive days were associated with mild toxicity and an induction of P1A. In our experiments, mice received only three

injections of 5-azaCdR, but at a higher dose (4 mg·kg⁻¹) and without obviously increased toxicity. Consistent with our *in vitro* findings, VPA (5 mM) was delivered to mice after the first two injections of 5-azaCdR. The mice were weighed daily, with no differences noted between the groups. Upon completion of the treatment, mice from the treated and untreated groups were killed, and tumour tissues were collected. All the results are summarised in table 1 and representative illustrations for each group are shown in figure 6. In mice from group 1 (control; n=10), the omentum was invaded by AK7 cells, and constituted a hard, white-coloured tissue that was easily isolated from the brown/yellowish soft pancreatic tissue. The tumour mass varied greatly between untreated animals (mean \pm SEM 261.7 \pm 43.5 mg), and the presence of lymphocytes was rare. In animals from group 2 (n=6), which received only 5-azaCdR, the tumour mass was slightly reduced (199.2 \pm 32.5 mg) and some areas showed accumulation of non-organised lymphocytes. With VPA alone (group 3; n=6), the tumour mass was even more reduced (135 \pm 18.7 mg) and important foci of lymphocytes

TABLE 1 Staging of omental invasion by the tumour and lymphocyte infiltration

	Tumour mass mg	AK7 tumour/omental tissue [#]	Lymphocyte aggregates [†]
Control [‡]	261.7 ± 43.5	++++/-	0
5-azaCdR	199.2 ± 32.5	+++/+	<1
VPA	135 ± 18.7	++/++	3
5-azaCdR/VPA	99.8 ± 22.1*	++/++	6

Data are presented as mean ± SEM, unless otherwise stated. 5-azaCdR: 5-aza-2'-deoxycytidine; VPA: valproic acid. [#]: degree of omental tissue invasion by AK7 tumour cells. ++++/-: omentum totally occupied by tumour cells, no residual fat cells; +++/+ : residual fat cells in the central part of the omentum; ++/++: equivalent amounts of AK7 and fat cells in the omentum. [†]: quantification of lymphoid cell infiltrates as the average number of lymphocyte aggregates per section of tumour in the whole group of mice; an aggregate was defined as a dense, organised accumulation of lymphoid cells covering an area >225 × 300 μm². [‡]: vehicle. *: p<0.05.

were observed (containing several regulatory T-cells labelled with Foxp3). Finally, mice receiving the combination of 5-azaCdR/VPA (n=6) showed the most significant reduction in tumour mass (99.8 ± 22.1 mg; p<0.05) and a greater number of lymphocyte aggregates. However, in contrast to group 3, tumour cells were excluded from lymphocyte foci, which contained rare Foxp3+ cells localised at the periphery.

In a second series of experiments, we determined whether 5-azaCdR/VPA treatment induces a specific T-cell response against AK7 cells. We isolated CD8a+ T-cells from the spleens of mice having received, or not, the combined treatment (n=5 for both). We then stimulated these CD8a+ purified T-cells with AK7 tumour cells (fig. 7a). As determined by ELISPOT assay, the number of IFN-γ-secreting cells was greater in spleens from 5-azaCdR/VPA-treated mice: mean ± SEM 80 ± 6 spots per 5 × 10⁵ cells compared with 18 ± 4 spots per 5 × 10⁵ cells for untreated mice, suggesting the induction of a tumour cell-specific immune response by the treatment. Furthermore, this response was mainly directed towards treatment-induced tumour antigens. Indeed, the IFN-γ response doubled with CD8a+ T-cells from the spleens of treated mice stimulated with AK7 cells that were previously treated with 0.5 μM 5-azaCdR for 72 h and 5 mM VPA for 48 h (154 ± 9 spots per 5 × 10⁵ cells), as compared with untreated AK7 cells (80 ± 6 spots per 5 × 10⁵ cells). No spots were observed in wells containing medium alone and 12 ± 4 spots per 5 × 10⁵ cells were observed in wells containing only T-cells. Most importantly, we succeeded in isolating CD8a+ lymphocytes from the tumours of mice having received the 5-azaCdR/VPA treatment, but not from untreated animals. As for the experiment performed with splenocytes, we observed an increase in the number of T-cells producing IFN-γ in response to treated AK7 cells (28 ± 2 spots per 1 × 10⁵ cells), as compared with untreated AK7 cells (10 ± 2 spots per 1 × 10⁵ cells) (fig. 7b) and medium alone (4 ± 2 spots per 1 × 10⁵ cells).

DISCUSSION

Hypomethylating drugs and HDAC inhibitors are used clinically for their ability to promote growth arrest, differentiation and apoptosis of tumour cells [13, 30]. Decitabine (5-azaCdR), an inhibitor of DNMTs, is the most commonly used hypomethylating drug [31]. Depakine (VPA) and vorinostat (SAHA) are two HDAC inhibitors currently being evaluated for MPM treatment [8, 9, 14]. The histone acetylation mechanism involves two enzyme families: HATs and HDACs. HATs transfer acetyl groups to amino-terminal lysine residues in

histones, whereas HDACs catalyse the removal of acetyl groups, leading to chromatin condensation and transcriptional repression [32, 33].

Apart from their intrinsic toxic properties, our work demonstrated that epigenetic drugs used alone, or in combination, could have an impact on the expression of TAAs and, therefore, could improve the recognition of tumour cells by cytotoxic T-cells. We focused our attention on CTAs, which are of growing interest as immunotherapeutic targets because of their *in vivo* immunogenic properties, expression among tumours of different types and absence in normal tissues [28, 34].

Consistent with a previous observation made by SIGALOTTI *et al.* [19], we showed that 5-azaCdR used alone can induce or increase the expression of NY-ESO-1, MAGE-A1 and MAGE-A3 in MPM cells of the epithelioid subtype at doses that are not cytotoxic. In contrast to 5-azaCdR, its close analogue 5-azacytidine (vidaza), which is preferentially incorporated into RNA but not DNA, was not efficient in inducing the expression of the CTAs analysed (data not shown). Conversely, VPA or SAHA alone displayed relatively high toxicity on these cells but did not affect CTA expression. However, we showed that these two drugs, used at doses below optimal toxicity, enhanced CTA expression in MPM cells pre-treated with 5-azaCdR. In addition, the overall toxicity of the combined treatment was slightly increased when compared with the HDAC inhibitor alone. In proliferating cells, pre-treatment with 5-azaCdR induces demethylation of the CpG islands located in the promoters of silenced genes, such as those encoding CTA, as reported for NY-ESO-1 in a glioma cell model [35], and, as a consequence, allows their expression. Thus, the subsequent use of VPA or SAHA, which can directly inhibit HDAC activity and leave the chromatin in an open conformation, enhances the expression of CTAs induced by 5-azaCdR through a better accessibility of transcription factors to DNA. Importantly, NMCs treated similarly did not express CTAs, such as NY-ESO-1, suggesting that such treatment did not affect the immunogenic status of normal, nonproliferating cells.

Furthermore, we observed that all HLA-A*0201+ MPM cell lines treated with 5-azaCdR/VPA (or 5-azaCdR/SAHA) can be recognised by a CD8+ T-cell clone that is HLA-A*0201-restricted and specific to NY-ESO-1(157–165). Hence, *de novo* synthesised NY-ESO-1 antigen is functionally processed and

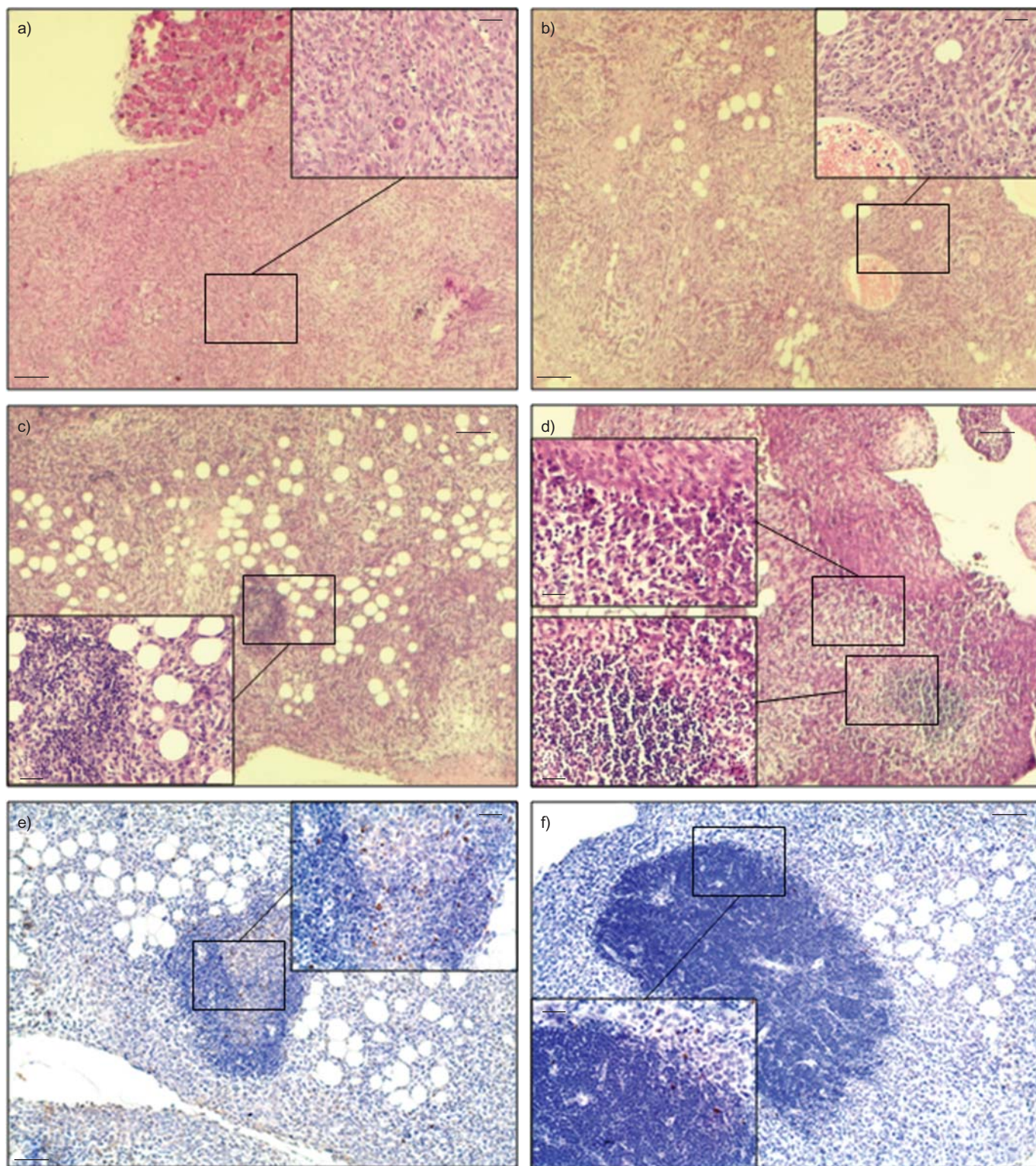


FIGURE 6. Histopathological examination of AK7 tumours implanted in mice. 5×10^6 AK7 murine mesothelioma cells were injected intraperitoneally into C57BL/6 mice. Mice from a) group 1 (n=10) had no treatment. Mice from groups b) 2 (n=6) and d) 4 (n=6) received three injections of 5-aza-2'-deoxycytidine (5-azaCdR) ($4 \text{ mg} \cdot \text{kg}^{-1}$). Valproic acid (VPA) (5 mM) was added to the drinking water of mice from groups c) 3 (n=6) and d) 4. a–d) Tumour sections stained with haematoxylin–eosin–safran. e, f) Immunohistochemical staining of tumour sections from mice treated with VPA e) alone or f) in combination with 5-azaCdR, using anti-Foxp3 antibodies. Foxp3+ cells appear with a brownish nuclear staining. Scale bars=250 μm (inset 40 μm) (magnification $4 \times$ (inset $20 \times$)).

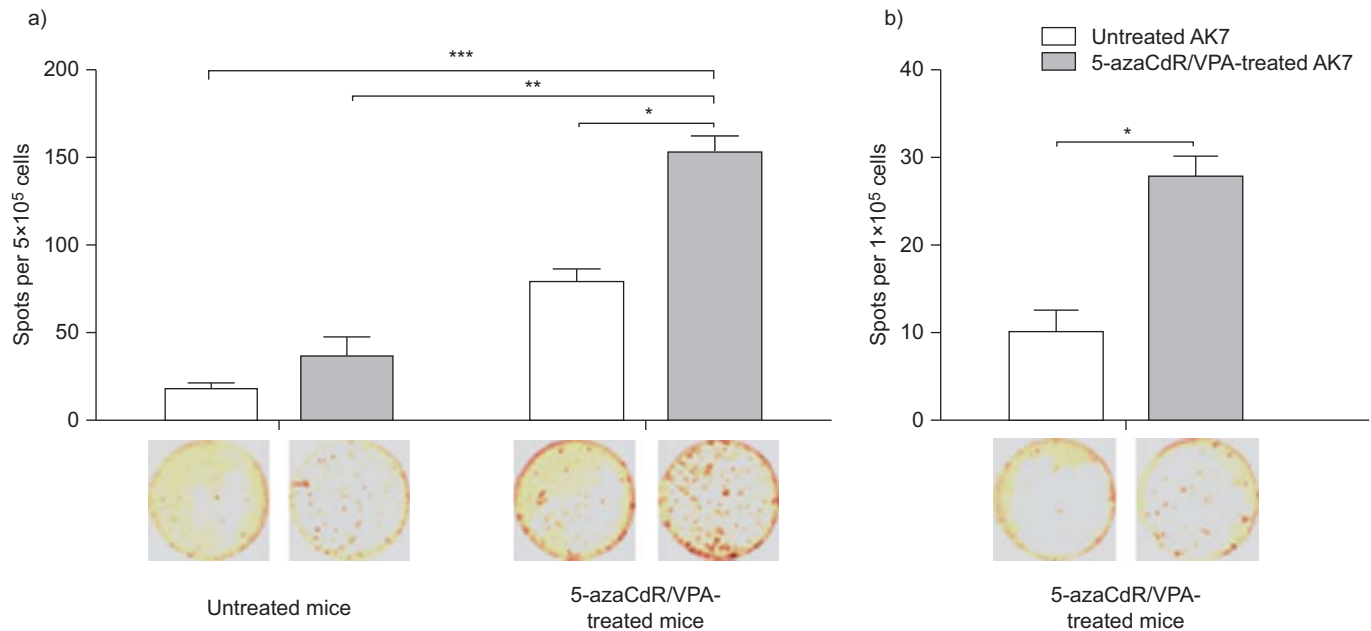


FIGURE 7. T-cell antitumour response *in vivo* after 5-aza-2'-deoxycytidine (5-azaCdR)/valproic acid (VPA) treatment. CD8a+ T-cells from the a) spleen and b) CD8a+ tumour-infiltrating T-cells from C57BL/6 mice bearing tumours (treated or not with 5-azaCdR/VPA) were stimulated for 36 h with untreated AK7 cells or 5-azaCdR/VPA-treated AK7 cells. Secretion of interferon- γ by CD8a+ cells was measured using an ELISPOT assay. The mean \pm SEM number of spots in triplicate wells was calculated and expressed as number of spots per 5×10^5 or 1×10^5 CD8a+ cells. Significant differences in response were determined using a) Kruskal–Wallis ANOVA followed by Dunn's post-test, or b) Mann-Whitney test. A representative well for each condition is displayed under the corresponding bar of the histogram. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

presented on the tumour cell surface through major histocompatibility complex class I molecules. Consequently, we have shown that, in addition to the cytotoxicity induced by the HDAC inhibitor, the sequential 5-azaCdR/VPA treatment induces a specific lysis of MPM cell lines by NY-ESO-1-specific CD8+ T-cells.

Finally, using a murine model of mesothelioma, we demonstrated that such sequential association of treatment with 5-azaCdR and VPA could inhibit tumour progression by $\sim 60\%$ after 3 weeks. This effect is due to both the toxicity of the treatment itself (indicated by active caspase-3 staining within the tumours of treated mice (data not shown)) and also to the activation of an antitumour immune response. Indeed, although AK7 cells are known to secrete a large amount of transforming growth factor (TGF)- β and to promote tolerance towards the tumour [36], we saw large lymphocyte infiltrates within the tumours of treated mice but few Foxp3+ cells. Furthermore, we showed that pools of CD8a+ T-cells isolated from the tumours (and also from the spleens) of treated, compared with untreated, mice contained more T-cells able to recognise AK7 cells. As IFN- γ has opposite immunomodulatory effects to TGF- β , this cytokine may play an important role in the antitumour response observed after 5-azazCdR/VPA treatment.

Although various clinical trials have already shown antitumour activity of 5-azaCdR, SAHA or VPA alone in leukaemias [3, 6, 7, 11], very few data are available concerning the activity of these drugs in patients with MPM [4]. SCHRUMP *et al.* [4] evaluated the feasibility and toxicity of continuous infusion of 5-azaCdR alone for 72 h in 35 patients with lung and

oesophageal cancers or MPM in a phase I clinical trial. The authors determined that the maximum tolerated dose of 5-azaCdR was $60\text{--}75 \text{ mg}\cdot\text{m}^{-2}$ and, interestingly, that prolonged infusion induced CTA gene expression (NY-ESO-1 and MAGE-A3) in 36% of patients.

SAHA is the first HDAC inhibitor approved for the treatment of cancer, in particular cutaneous T-cell lymphoma [5]. Its safety is being evaluated in numerous phase I clinical trials for the treatment of solid tumours. Collected safety data showed that the use of SAHA in combination therapies, most commonly at 400 mg daily for 14 days, is generally well tolerated, and preliminary evidence of anticancer activity has been observed across a range of malignancies [11]. Interestingly, in a phase I study, two out of 13 patients with advanced MPM who had progressed after first-line chemotherapy demonstrated a partial clinical response after oral administration of SAHA for several cycles of therapy [9].

Among HDAC inhibitors, VPA is known to be less active than hydroxamic acids, such as SAHA, but its pharmacokinetic properties and bioavailability favour its use as a clinical treatment. For instance, VPA has a longer half-life than SAHA (16–17 h and 19–47 min, respectively) and its widespread use as an antiepileptic drug over several decades has proven that its side-effects are acceptable in the long term. VPA is currently being tested as an anticancer agent, either alone or in combination with other agents, mostly in haematological diseases [7]. In a phase I/II study, 53 patients with advanced leukaemia were treated with a fixed dose of 5-azaCdR ($15 \text{ mg}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ *i.v.* for 10 days) administered concomitantly with $50 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ VPA, given orally for 10 days [37]. This

combined 5-azaCdR/VPA treatment was shown to be safe and active, and was also associated with significant DNA hypomethylation, induction of histone acetylation and gene reactivation. Clinical studies with VPA alone or in combination with other agents have also been performed on solid tumours [38]. For example, in a phase I study, patients with advanced solid tumours were treated with VPA by intravenous infusion: consistent with the observations in leukaemia patients, doses $\leq 60 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ for five consecutive days were well tolerated and showed detectable biological activity, *i.e.* accumulation of hyperacetylated H3 and H4 histones in cell lysates of peripheral blood lymphocytes [39]. In the context of MPM, it has been shown recently that VPA improves the proapoptotic effect of pemetrexed/cisplatin, the usual first-line treatment regimen for this tumour, in tumour cells from patients' biopsies and in a mouse model of MPM [8]. The clinical benefit of VPA plus doxorubicin was recently evaluated in a phase II trial (see protocol 01062 at www.elcwp.org). There are currently very few data available concerning the use of 5-azaCdR and VPA combinations (sequential or concomitant) for the treatment of solid malignancies. Only one clinical trial (phase I) for the treatment of patients with nonsmall cell lung cancer is ongoing (see protocol NCT00084981 at www.clinicaltrials.gov). Notably, in all these studies, patients received 5-azaCdR, SAHA or VPA by intravenous infusion or orally at doses below those used here. However, other administration protocols can be proposed, such as infusion into the pleural cavity in direct contact with MPM tumour cells. This route of injection, which has been used successfully in clinical trials with interleukin-2 [40], may allow the use of higher doses of drugs that are incompatible with systemic administration and may also diminish side-effects in other organs.

Altogether, these clinical trials have allowed the measurement of 5-azaCdR, SAHA and VPA toxicities but, considering our results presented here, the impact of these molecules on the immune response towards cancer cells should be evaluated further. Our investigations have demonstrated that sequential 5-azaCdR/VPA or 5-azaCdR/SAHA treatments represent potential new strategies to induce MPM cell death and to modulate the immunogenicity of remaining, drug-resistant tumour cells. This work opens up new opportunities for the development of additional MPM treatment strategies that use chemotherapeutic drug combinations, such as 5-azaCdR/VPA or 5-azaCdR/SAHA, to induce MPM cell apoptosis and neo-expression of CTAs, such as NY-ESO-1, in the remaining, apoptosis-resistant tumour cells. These latter cells then become targets for a cytotoxic and TAA-specific immune response. Furthermore, this strategy may bypass the development of antigen-specific tolerance mechanisms that occur when CTAs are expressed by cancer cells during tumour progression. The association of such chemotherapeutic with immunotherapeutic treatments should be evaluated in the future.

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STATEMENT OF INTEREST

None declared.

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