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5-Aza-2′-deoxycytidine potentiates antitumour immune response induced by photodynamic therapy

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Abstract

Photodynamic therapy (PDT) of tumours is based on administration of a photosensitiser followed by irradiation of the tumour with visible light leading to production of reactive oxygen species that cause direct tumour cell death and vascular damage. PDT also initiates acute local inflammation, which facilitates the development of adaptive antitumour immunity. It has recently been reported that PDT can induce strong antitumour immunity towards tumours cells expressing P1A, tumour-associated antigen. Using four different tumour models, we show that antitumour immune response can be further improved when PDT is combined with a clinically approved epigenetic agent that induces expression of a silenced P1A antigen. Induction of P1A with 5-aza-2′-deoxycytidine, a methyltransferase inhibitor, resulted in potentiated antitumour effects in mice with Lewis lung carcinoma and 4T1 mammary carcinoma when combined with PDT treatment. In CT26 colon carcinoma and EMT6 mammary carcinoma models the combination therapy resulted in complete responses and long-term survival. All long-term surviving mice were resistant to re-inoculation with the same tumour cells. Antitumour efficacy of the combination treatment was severely impaired by depletion of $CD8^+$ cytotoxic T cells, whereas adoptive transfer of $CD8^+$ T cells from long-term surviving mice allowed for significant tumour growth delay in tumour-bearing mice. Taken together, these findings show that PDT leads to strong specific antitumour immune responses, and that epigenetic modification of tumour antigens levels may be a novel

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None declared.

approach to further enhance the effectiveness of PDT. The present results provide a strong rationale for clinical development of this therapeutic approach.

Keywords

5-Aza-2′-deoxycitidine; Cancer; Photodynamic therapy; Specific antitumour immunity; Tumour associated antigens

1. Introduction

Photodynamic therapy (PDT) is a successful, clinically approved and minimally invasive therapeutic procedure used in the treatment of several types of solid tumours. It involves administration of a photosensitiser that accumulates in tumour tissue, followed by illumination with a visible light. Light activated photosensitiser, in the presence of molecular oxygen, generates singlet oxygen and cytotoxic reactive oxygen species, that cause oxidative damage to intracellular macromolecules leading to tumour cell death [1]. Antitumour effects of PDT result from the combination of mechanisms involving direct cytotoxicity to tumour cells, destruction of tumour vasculature and induction of acute local inflammatory response. The latter may lead to the development of antitumour and antigen-specific immune response [2–4].

PDT causes oxidative stress at irradiation site leading to massive photooxidative damage in cancer cells, tumour vasculature and stroma. PDT-associated local tissue injury leads to disruption of tumour microenvironment and tissue integrity and homoeostasis [5] evoking a host response in the form of an acute inflammatory reaction. Importantly, PDT causes surface exposure or release of damage-associated molecular patterns (DAMPs) from tumour cells that trigger activation of dendritic cells [6], capable of priming adaptive immune responses. After antigen capture and recognition of DAMPs, dendritic cells (DCs) become activated and migrate to local lymphoid organs, where they mature and present antigen-derived peptides in association with major histocompatibility complex (MHC) molecules to T lymphocytes. This results in activation of $CD4^+$ T helper (Th) cells and $CD8^+$ cytotoxic T cells (CTLs), B cells and initiation of the adaptive immune response [7].

Ideally, anticancer treatment would lead to eradication of primary tumour, as well as to induction of specific antitumour immune response allowing for the control of distant metastases and protection from tumour relapse. It was shown that long-term antitumour effects of PDT depend on the presence and activity of adaptive immunity [8]. However, in many tumour models PDT alone is insufficient to induce robust immune response that would lead to complete tumour eradication and long-term control of distant metastases. Therefore, combining PDT with other antitumour therapies especially those modulating immune responses is of high clinical significance. Previous studies revealed that PDT could induce robust adaptive immunity towards tumour cells expressing strong, exogenous antigens, such as β-galactosidase [4] or green fluorescent protein [9]. All mice bearing antigen-positive tumours were cured and became resistant to re-challenge. However, antigen-negative tumours were resistant to PDT-induced immunity. Recently, we have shown that PDT can also induce strong antigen-specific antitumour immunity towards

tumours expressing P1A antigen, the mouse homologue of human melanoma associated antigen (MAGE), a type of tumour-associated antigen (TAA) [10] providing strong rationale for developing combination therapies that can enhance this effect. One of the therapeutic approaches that has been suggested to be able to restore/enhance the expression of TAA is epigenetic modification [11].

Epigenetic mechanisms that involve DNA methylation, act as regulators of gene expression. In tumour cells the epigenetic modification and aberrant silencing of numerous genes is one of the most frequently observed molecular changes [12], that plays an important role in tumour evasion from immune surveillance. It has been shown that epigenetic modifications are responsible for down-regulation of expression of both MHC class I molecules and TAA [13,14]. DNA methylation can be reversed with chemical agents such as methyltransferase inhibitor, 5-aza-2′-deoxycitidine (5-aza-dC), a clinically approved drug for treatment of myelodysplastic syndrome and acute myeloid leukaemia. Use of 5-aza-dC can restore expression of silenced or down-regulated antigens in tumours leading to the higher release of TAA in the PDT-treated microenvironment [11]. The immune system is able to recognise TAA presented by MHC class I molecules on the tumour cell surface, and the CTLs can subsequently destroy these cancer cells via perforin and granzyme attack [15]. Thus, modifying DNA methylation by 5-aza-dC can potentially enhance the effectiveness of antitumour therapies that lead to activation of the immune system. By restoring the levels of TAA and MHC molecules in tumour cells the epigenetic reversal agents may contribute to better antigen presentation and facilitate tumour recognition by immune cells leading to effective and antigen-specific antitumour immunity.

In the current study we followed up on our recent work showing that PDT can lead to development of anti-P1A antigen specific immune response. We show for the first time that pre-treatment with the 5-aza-dC can lead to restoration of P1A expression in wild type P1A-negative tumours, and in combination with PDT can exert long-term antitumour effects. Since we failed to demonstrate induction of specific anti-P1A immune response in the combination group, expression of P1A can be regarded as a surrogate marker representing a possibly larger group of induced TAA that become targets of the immune response.

2. Materials and methods

2.1. Cell cultures

Epithelial mammary carcinoma (EMT6 and 4T1) and colon carcinoma (CT26) cell lines were purchased from American Type Culture Collection, ATCC (Manassas, VA, USA). Lewis lung carcinoma (LLC) cell line was purchased from European Collection of Cell Cultures, ECACC (Porton Down, Salisbury, UK). Cells were cultured in Roswell Park Memorial Institute (RPMI 1640) medium (CT26 and 4T1) or Dulbecco's modified Eagle's medium (EMT6, LLC) supplemented with 10% heat-inactivated foetal bovine serum (Hyclone) and antibiotic/antimycotic solution (Sigma, St. Louis, MO). Cells were cultured under standard conditions in a 5% CO_2 humidified incubator at 3)7 °C.

2.2. In vivo studies

For all experiments 8–12-week-old BALB/c or C57BL/6 females with an average weight of 19 g were used. Mice were obtained from the Animal House of the Polish Academy of Sciences, Medical Research Center (Warsaw, Poland). All *in vivo* experiments were performed in accordance with the guidelines approved by the Ethics Committee of the Medical University of Warsaw.

2.3. Reagents

Photofrin (Axcan Pharma Inc., Houdan, France) was used as a photosensitiser. For *in vitro* studies Photofrin was dissolved in Dulbecco's modified Eagle's medium and for *in vivo* studies Photofrin was reconstituted in 5% glucose. 5-aza-dC was purchased in Sigma–Aldrich and dissolved in 0.9% NaCl. Diluents were used as controls.

2.4. Reverse transcription polymerase chain reaction (RT-PCR)

Cells were washed with phosphate buffered solution (PBS) and lysed in 0.5 ml of TRIzol reagent (Invitrogen) and the total RNA was isolated according to modified Chomczynski method. Concentration and purity of RNA were determined with a NanoDrop ND-2000c spectrophotometer (Thermo Scientific). For cDNA synthesis 1 μg of RNA was used with oligo(dT) primer and Avian Myeloblastosis Virus (AMV) reverse transcriptase (EURx). PCR was performed using Mastercycler personal (Eppendorf) and Color OptiTaq polymerase (EURx) or OneTaq® 2× Master Mix (NEB). Products of PCR amplification were analysed by electrophoresis in ethidium bromide stained 1% agarose gel, visualised under UV light and photographed using Alpha Imager (Cell eBioscence). The primer sequences were as follows: GAPDH forward – 5′-CCCTTCATTGACCTCAACT ACATGG-3′ and GAPDH reverse – 5′-CCTGCTTCA CCACCTTCTTGATGTC-3′; P1A forward – 5′-CGGA ATTCTGTGCCATGTCTGATAACAAGAAA-3′ and P1A reverse – 5′-CGTCTAGATTGCAACTGCATGC CTAAGGTGAG-3′.

2.5. Cell isolation and re-stimulation

Spleens and tumour draining lymph nodes (LNs) (popliteal, inguinal, axillary, brachial) were isolated from mice of each experimental group. To obtain single cell suspension, spleens and LNs were forced through a 70 μm cell strainer. In order to lyse erythrocytes, cells were incubated for 5 min in 37 °C in 150 mM NH_4Cl, 1 mM $NaHCO_3$, pH 7.4 buffer. Next, cells were washed and resuspended in RPMI 1640 medium with 10% foetal bovine serum. Cells were then counted, stained with antibodies and analysed in flow cytometry. For *ex vivo* stimulation 2×10^6 cells were seeded on 96-well plate and incubated in RPMI supplemented with 10% foetal bovine serum and antibiotic/antimycotic solution for 6 h at 37 °C with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma–Aldrich) and 2 mg/ml ionomycin (Sigma–Aldrich). GolgiPlug (BD Biosciences) was added during the last 4 h of culture. After incubation time plate was placed in 4 °C overnight.

2.6. Flow cytometry

2.6.1. In vitro staining—After a 72 h incubation with 5-aza-dC tumour cells were trypsinised, washed with PBS and incubated with anti-$H2k^d$ or anti-$H2k^b$ antibodies (mAbs)

coupled with fluorescein isothiocyanate (FITC) (BD Bioscience) in PBS containing 1% bovine serum albumin (BSA) for 30 min in 4 °C. Cells were analysed in FACScan (Becton Dickinson) using CellQuest Pro Software Version 5.2.

2.6.2. Ex vivo staining—After isolation and re-stimulation with PMA and ionomycin, cells were stained with anti-CD4 conjugated with PerCp-Cy5 and anti-CD8 coupled with FITC mAbs. Subsequently, cells were fixed and permeabilised using Cytofix/Cytoperm, Perm/Wash buffers (BD Biosciences) according to the manufacturer's instructions. For intracellular staining anti-interferon-γ (IFN-γ) conjugated with phycoerythrin (PE) and anti-interleukin-17 (IL-17) coupled with allophycocyanin (APC) mAbs were used. In the co-culture activation assay splenocytes were stained with anti-CD8-FITC and anti-CD107-PerCp-Cy7 mAbs as described above. Cells were analysed on FACSAria using Diva software. All antibodies were purchased from BD Biosciences or eBioscience.

2.7. Co-culture activation assay

Isolated splenocytes (5×10^6) were seeded into 48-well plates and co-cultured with 1×10^6 5-aza-dC-treated EMT6 target cells. After 3 days of culture, cells were re-stimulated for 6 h as described above, stained with antibodies and analysed in flow cytometry.

2.8. Adoptive transfer

$CD8^+$ T cells were magnetically purified by negative selection (MiltenyiBiotec) from combined lymph nodes and spleens of naïve or long-term surviving mice. Efficiency of selection was determined by flow cytometry, and 7×10^6 $CD8^+$ T cells were immediately transferred into BALB/c tumour bearing mice by intravenous (i.v.) injection. 3 days before adoptive transfer, mice were subcutaneously inoculated with EMT6 cells into the right thigh (1×10^5 cells), and treated with 5-aza-dC. Tumour growth was monitored three times a week.

2.9. Lymphocyte depletion

Mice were injected i.p. with 100 μg of anti-CD4 (GK1.5) or anti-CD8 (YTS169) mAbs on day 4, 11 and 18 of the experiment. Control mice received 100 μg of iso-type control mAb. Level of depletion was evaluated 48 h after each mAbs injection. Blood samples were collected from cheek vein of experimental mice, stained with anti-CD4 or anti-CD8 mAbs and analysed by flow cytometry.

2.10. Tumour treatment and monitoring

Before inoculation, tumour cells were harvested, washed twice and resuspended in PBS. Viability (>95%) of tumour cells, was verified by trypan blue staining. 3×10^5 tumour cells (in 30 μl) was inoculated subcutaneously into the right thigh of experimental mice. On days 1–3 mice were treated intraperitonally (i.p.) with 5-aza-dC (0.8 mg/kg for EMT6, CT26, 4T1 and 0.4 mg/kg for LLC tumour models). Photofrin was administered i.p. at a dose of 10 mg/kg (on day 5 for EMT6, 4T1 and CT26 or on day 7 for LLC tumour models). 24 h later tumours were illuminated with 630 nm light delivered by He-Ne ion laser (Laser instruments, Warsaw, Poland) through the optical fibre. The power of the laser was 47

mW/cm^2 and the total fluence was 65 J/cm^2 for EMT6 and 4T1, 55 J/cm^2 for CT26 and 85 J/cm^2 for LLC. During the time of illumination mice were anesthetised and restrained. Tumour growth was monitored three times per week as described previously [16,17]. Mice were ethically sacrificed when any of the tumour diameter reached 15 mm.

2.11. Statistical analyses

Differences in *in vitro* studies and tumour volumes were analysed for significance by Student's *t* test with significance level set at $p < 0.05$. The nature of the interaction (synergy, additivity or antagonism) observed between 5-aza-dC and PDT was analysed using the Calcusyn software (Biosoft, Cambridge, England), which uses the combination index (CI) method of Chou and Talalay, based on multiple drug effect equation. For *ex vivo* studies significance was calculated by Mann–Whitney test. Survival rate of animals was analysed for significance by log-rank survival analysis. Significance was defined as a two-sided $p < 0.05$.

3. Results

3.1. 5-aza-2′-deoxycytydine induces expression of P1A and modulates the levels of MHC class I molecules in tumour cells

P1A expression was determined with RT-PCR in P1A-negative EMT6, 4T1, CT26 and LLC cell lines. As shown in Fig. 1 induction of P1A expression was time and dose dependent in cells incubated with 5-aza-dC. Flow cytometry analyses revealed that 5-aza-dC also increased the levels of H2k^d molecules in EMT6 and 4T1 cells, that have a low constitutive expression of MHC class I molecules and induced H2k^b in MHC class I negative LLC tumour cells (Fig. 2). The effects of 5-aza-dC on MHC class I levels in CT26 cells were only statistically significant at the highest dose tested.

Subsequently we determined induction and duration of P1A expression *in vivo* in EMT6, CT26, 4T1 and LLC tumours. Mice were treated for 3 days with 0.4 or 0.8 mg/kg/day of 5 -aza-dC. After further 7, 14, 18 or 21 days mice were sacrificed and mRNA levels for P1A gene were determined in tumour tissue using RT-PCR. Fig. 3 shows that 5-aza-dC can induce *de novo* expression of P1A antigen in all tumours. The expression of P1A persisted for at least 21 days in EMT6 tumours, 18 days in LLC tumours, and 14 days in 4T1 and CT26 tumours.

3.2. 5-aza-2′-deoxycitidine potentiates antitumour effects of PDT in vivo

Considering recent studies suggesting that *de novo* induction of tumour antigens in solid tumours may allow for reversal of tumour escape mechanisms [11], and the fact that constitutive expression of P1A antigen leads to development of antitumour immune response triggered by PDT [10], we investigated whether Photo-frin-PDT treatment combined with a three-day 5-aza-dC pre-treatment of P1A-negative wild type tumours to induce *in vivo* P1A expression can lead to immune-mediated, antitumour effects. To this end we used four different tumour models syngeneic with two strains of mice. Two doses of 5-aza-dC were used: 0.8 mg/kg in BALB/c mice (inoculated with EMT6, CT26 and 4T1 cells) and 0.4 mg/ml in C57/BL6 mice syngeneic with LLC cells. While complete responses were

obtained in combination groups in mice bearing EMT6 and CT26 tumours, a significantly prolonged survival was observed in mice bearing 4T1 and LLC tumours (Fig. 4). No tumours appeared for at least 90 days of observation in 80% and 71% of animals inoculated with EMT6 and CT26 cells, respectively. All tumour free mice rejected tumour re-challenge (untreated EMT6 cells) on day 95 into the contralateral thigh. Again, no regrowth of the tumours was observed for an additional 40 days. However, none of the long-term surviving mice that eliminated EMT6 tumour cells were capable of rejecting tumour cells of different origin (untreated 4T1 cells) (Supplementary Fig. 1). Of note, neither PDT nor 5-aza-dC treatment alone showed any significant therapeutic effects against 4T1 and LLC tumours, while in the case of EMT6 and CT26 tumours 20% of mice treated with PDT alone survived for over 90 days of observation. The antitumour effects of combination treatment did not result from direct potentiation of PDT-mediated cytotoxicity by 5-aza-dC. As shown in Supplementary Fig. 2, there were no synergistic cytostatic/cytotoxic effects of the combination treatment in any of the tested cell lines. Results were confirmed by Chou and Talalay calculations. The combination treatment was also very effective when 5-aza-dC was combined with PDT using a different photosensitiser, liposomal benzoporphyrin derivative mono acid ring A (BPD, Supplementary Fig. 3).

3.3. Antitumour treatment combining 5-aza-dC with PDT and activation of immune response

Two weeks after PDT, spleens and LNs were harvested from tumour bearing mice from all experimental group (8–9 mice per group). After *in vitro* re-stimulation with PMA and ionomycin, cytokine levels were determined by intracellular staining in $CD4^+$ and $CD8^+$ T cells with flow cytometry. A significant increase in LN $CD4^+$ and $CD8^+$ IFN-γ production was detected both in PDT and 5-aza-dC + PDT groups when compared with untreated controls and 5-aza-dC alone group. A similar trend was observed in splenocytes (Fig. 5A and B). In $CD4^+$ T cells, significantly increased IL-17 levels were observed in 5-aza-dC + PDT group in comparison with all other groups (Fig. 5C).

Furthermore, 3-day co-cultures of 5-aza-dC-treated (or non-treated) EMT6 target cells, with splenocytes isolated from groups of mice treated with either PDT alone or 5-aza-dC + PDT were established. Expression of CD107, a marker for cytolytic activity of T lymphocytes, was strongly upregulated in cells isolated from mice treated with PDT alone and with 5-aza-dC + PDT (Fig. 5D). However, no significant difference was observed between untreated control (no P1A expression), and 5-aza-dC-treated (with P1A expression) EMT6 cells when used as targets. Pentamers® staining for determination of P1A-specific $CD8^+$ T cell population in draining LNs and spleens revealed no significant changes between experimental groups. However, some increase in $CD8^+$ P1A-specific T cell population was detected in splenocytes isolated from mice treated with 5-aza-dC + PDT and co-cultured with EMT6 incubated with 10 μM 5-aza-dC (Supplementary Fig. 4C).

3.4. Long-term antitumour effect of 5-aza-dC combined with PDT is dependent on $CD8^+$ T cells

To investigate the involvement of different subsets of T cells in immune response mediated by combination of 5-aza-dC and PDT treatment, selective immunodepletion experiments

were carried out. Mice were injected i.p. with anti-CD4 or anti-CD8 mAbs to deplete the relevant target cells (Fig. 6A). Flow cytometry analysis of blood samples obtained from anti-CD8 and anti-CD4 mAbs treated mice showed that depletion efficiency was 99% for both $CD4^+$ and $CD8^+$ T cells (data not shown). The depletion of $CD8^+$ T cells in EMT6 tumour bearing mice completely abrogated the therapeutic effects of the combination treatment (Fig. 6B, right panel) without affecting tumour growth rate immediately after PDT (Fig. 6B, left panel). In contrast, 60% of mice that received isotype matched control Abs survived for over 60 days of observation. Treatment with anti-CD4 mAbs led to minor changes in survival rate in comparison with a group receiving isotype control mAbs (Fig. 6C). To confirm these observations in a different tumour model, experiments in CT26 tumour-bearing mice were carried out. Also in this model no long-term surviving animals were observed in combination group after depletion of $CD8^+$ T cells (Fig. 6D). In isotype control group more than 50% of mice survived without tumours for over 60 days.

To confirm the involvement of $CD8^+$ T cells in anti-tumour effects we performed adoptive transfer experiments. A single cell suspension of leukocytes was obtained from spleens and lymph nodes of naïve and long-term surviving mice and $CD8^+$ T cells were isolated by negative selection on magnetic beads. Flow cytometry analysis showed at least 90% pure $CD8^+$ T cell population (data not shown). The $CD8^+$ T cells isolated from spleens and LNs of naïve and long surviving PDT + 5-aza-dC treated mice (7×10^6/mouse) were adoptively transferred i.v. into EMT6 tumour-bearing mice pre-treated with 5-aza-dC as described above. A significant tumour growth delay was observed in mice with adoptively transferred $CD8^+$ T cells from long-term surviving mice in comparison with mice receiving $CD8^+$ T cells from naïve mice (Fig. 6E).

4. Discussion

This study reports for the first time that a clinically approved methyltransferase inhibitor 5-aza-dC can be used in combination with PDT to improve induction of adaptive immune response towards well-defined tumour antigen leading to the long-term survival of tumour-bearing mice. Although 5-aza-dC alone is capable of increasing MHC class I expression and inducing P1A (and possibly other antigens) [18], its antitumour effects are limited. Only when combined with PDT that leads to local tumour destruction and cytotoxic effects, the immunomodulatory activity of 5-aza-dC is revealed. Reduced or lost MHC class I expression on the tumour cell surface is one of the most common mechanisms of tumour immune escape [13]. Changes in MHC class I levels as well as in proteins involved in antigen presentation are mainly caused by epigenetic events [19]. Some tumours such as mastocytoma P815, Meth A sarcoma and J558 plasmacytoma express P1A constitutively [20,21]. However, most experimental tumours in mice are P1A-negative, possibly due to the process of immunoediting leading to selection of antigen-loss variants in immunocompetent mice. A recent study by Mroz et al. revealed that the presence of P1A in tumour cells is sufficient to induce systemic antitumour immunity after PDT [10]. The results shown here expand on those findings and provide some intriguing additional observations. For example, long-term surviving mice rejected re-challenge with the same tumour cells, even though the re-inoculated tumour cells were not pre-incubated with 5-aza-dC and were P1A-negative (Supplementary Fig. 1). This observation indicates that the presence of P1A is not necessary

to sustain long-term immunity. Moreover, the combination treatment is not statistically different from 5-aza-dC alone when the increased number of P1A-specific $CD8^+$ CTLs is measured (staining with Pentamers®, Supplementary Fig. 4A and B). Therefore, it is possible that not only P1A, but also other epigenetically silenced TAA are re-expressed after 5-aza-dC administration, and the combination therapy triggers P1A-independent immunity. Long-term surviving mice were not able to reject unrelated syngeneic tumours (Supplementary Fig. 1), even if these mice received 5-aza-dC in order to induce P1A (data not shown). This observation is in agreement with previous findings showing that effective immunisation against a single antigen can induce concomitant immunity to other TAA [22].

The mechanisms of PDT-induced antitumour immune response have been extensively studied during the past decade. Many reports support the role for $CD8^+$ T cells in antitumour efficacy of this treatment [23,24,25]. $CD8^+$ T cells are described as the key players in PDT mediated long-term control of tumours, while $CD4^+$ helper T cells seem to play a supportive role [1,26]. Those reports are in accordance with results from this study, where we showed that the presence of $CD8^+$ T cells is critical for eliciting antitumour effects, since the removal of these cells was associated with worse treatment outcome. The removal of $CD4^+$ T cells on the other hand only slightly affected antitumour efficacy of the combined treatment (Fig. 6). It was recently reported that $CD4^{+}$-IL-17^+ cells could induce antitumour immunity, leading to the eradication of established tumours. These cells do not reveal any killing activity, but are able to stimulate $CD8^+$ cytotoxic T lymphocytes [27]. IL-17 producing cells rapidly accumulate in the tumour-draining lymph nodes after PDT [28]. We observed a slight expansion of this subpopulation in mice treated with the combination of 5-aza-dC and PDT (Fig. 5).

Considering combination treatments with epigenetic drugs one should be aware of their potential limitations. Methyltransferase inhibitors and other epigenetic drugs are not selective and have been shown to restore the expression of various genes including oncogenes that might accelerate tumour progression [29].

In summary, we show that a well-designed chemotherapy regimen with 5-aza-dC restores/enhances the TAA and MHC class I expression levels in a set of mouse tumours, and when combined with PDT treatment that causes local tumour shrinkage and antigen release from treated tumour cells it leads to development of antitumour immune response and long-term survival. Moreover, the immune response elicited by the combination therapy leads to development of memory immunity and concomitant immunity that can protect from re-challenge with wild type P1A antigen-negative tumours. These findings provide a strong rationale for clinical application of this combination therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2014.01.017.

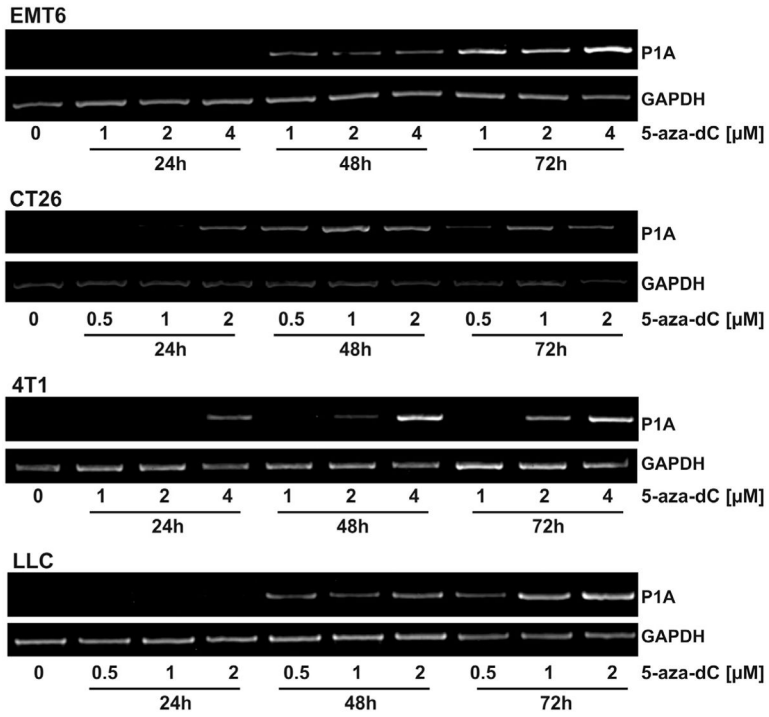

Fig. 1.

The influence of 5-aza-2′-deoxycitidine (5-aza-dC) on P1A expression in tumour cells. EMT6, CT26, 4T1 and Lewis lung carcinoma (LLC) cells were incubated for subsequent 24, 48 and 72 h with 5-aza-dC at indicated concentrations. After incubation time, cells were collected, total RNA was isolated and reverse transcribed into cDNA. mRNA levels for P1A gene were determined using reverse transcription polymerase chain reaction (RT-PCR) method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. PCR products were separated on 1% agarose gel, stained with ethidium bromide and visualised under UV light.

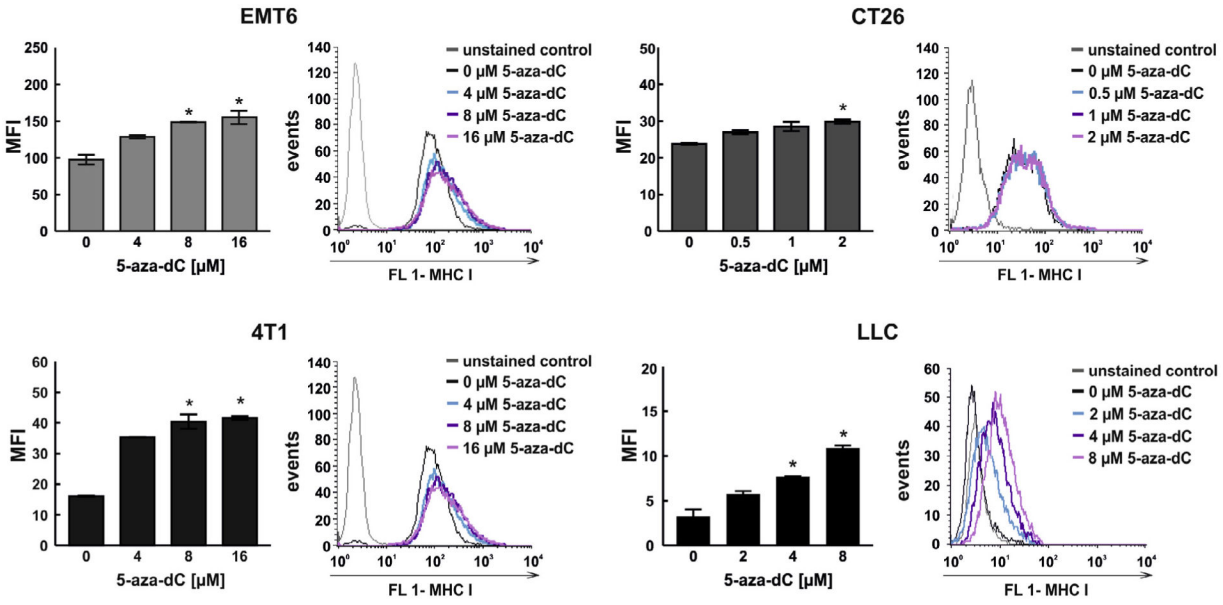

Fig. 2.

The influence of 5-aza-2′-deoxycitidine (5-aza-dC) on major histocompatibility complex (MHC) class I expression on the surface of tumour cells. EMT6, CT26, 4T1 and Lewis lung carcinoma (LLC) cells were incubated for 72 h with 5-aza-dC at indicated concentrations. After incubation time cells were collected and stained with fluorescein isothiocyanate (FITC) conjugated anti-$H2k^d$ (EMT6, CT26, 4T1) or anti-$H2k^b$ (LLC) mAbs. Prior to flow cytometry analysis, cells were washed and resuspended in phosphate buffered solution (PBS). Data show mean fluorescence intensity (±SD) relative to untreated controls (left) and representative histograms for each cell line (right). $^*p < 0.05$ as compared with controls in Student's t test.

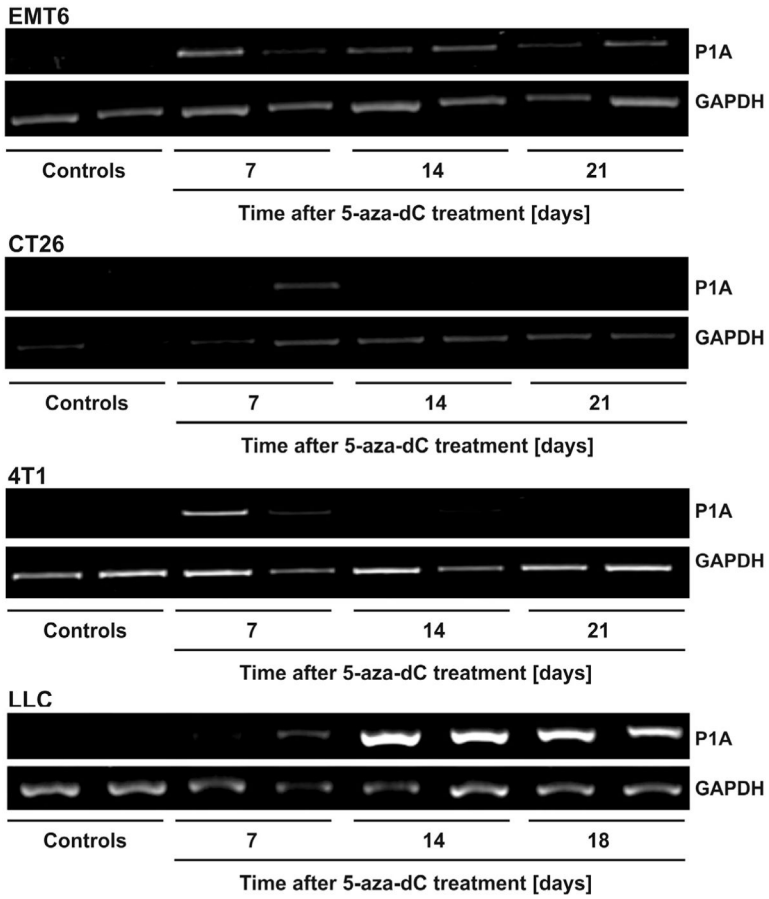

Fig. 3.

The influence of 5-aza-2′-deoxycitidine (5-aza-dC) treatment on P1A expression in tumours *in vivo*. EMT6, CT26, 4T1 and Lewis lung carcinoma (LLC) tumour cells were inoculated subcutaneously into the right thigh of experimental mice. Mice (n = 3–4) were treated for 3 days with 5-aza-dC (at dose of 0.8 mg/kg for EMT6, CT26, 4T1 and 0.4 mg/kg for LLC tumour models). At indicated time points after 5-aza-dC treatment mice were sacrificed and the total RNA was isolated from excised tumours. P1A and GAPDH (loading control) expression was determined at mRNA level using reverse transcription polymerase chain reaction (RT-PCR) method as described.

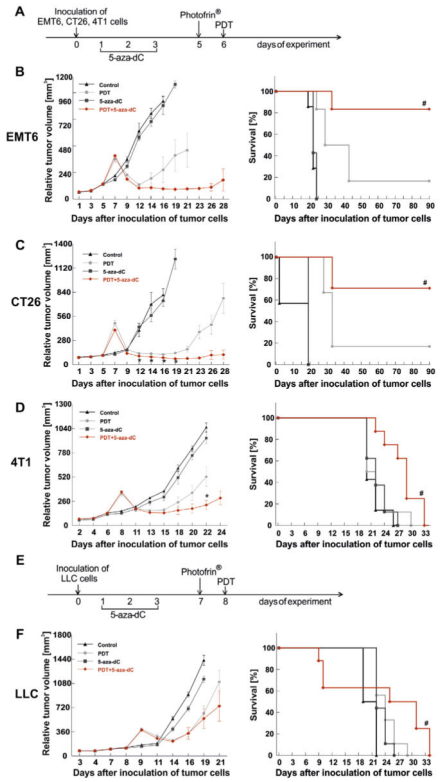

Fig. 4.

5-Aza-2′-deoxycitidine (5-aza-dC) potentiates antitumour effect of photodynamic therapy (PDT) in *in vivo* models. Mice were inoculated with 3×10^5 EMT6 (B), CT26 (C), 4T1 (D) or Lewis lung carcinoma (LLC) (F) cells. On days 1–3 mice were treated with 5-aza-dC (at a dose of 0.8 mg/kg for EMT6, CT26, 4T1 and 0.4 mg/kg for LLC tumour models). Photofrin was administered i.p. at a dose of 10 mg/kg on day 5 (B–D) or day 7 (F) of experiment, and 24 h later, the tumour site was illuminated with laser light at fluence of 65 J/cm^2 for EMT6 and 4T1, 55 J/cm^2 for CT26 and 85 J/cm^2 for LLC. Graphs (A and E) show detailed experimental scheme. Left panels represent mean tumour volumes (±SE) while right panels represent Kaplan–Meyer plots of the survival of mice bearing EMT6 (B, n = 6–7), CT26 (C, n = 6–7), 4T1 (D, n = 8) and LLC (F, n = 7–9) tumours. $^{*}p < 0.05$, compared with all other groups (Student's t test). $^{\#}p < 0.05$, compared with all other groups (log-rank test).

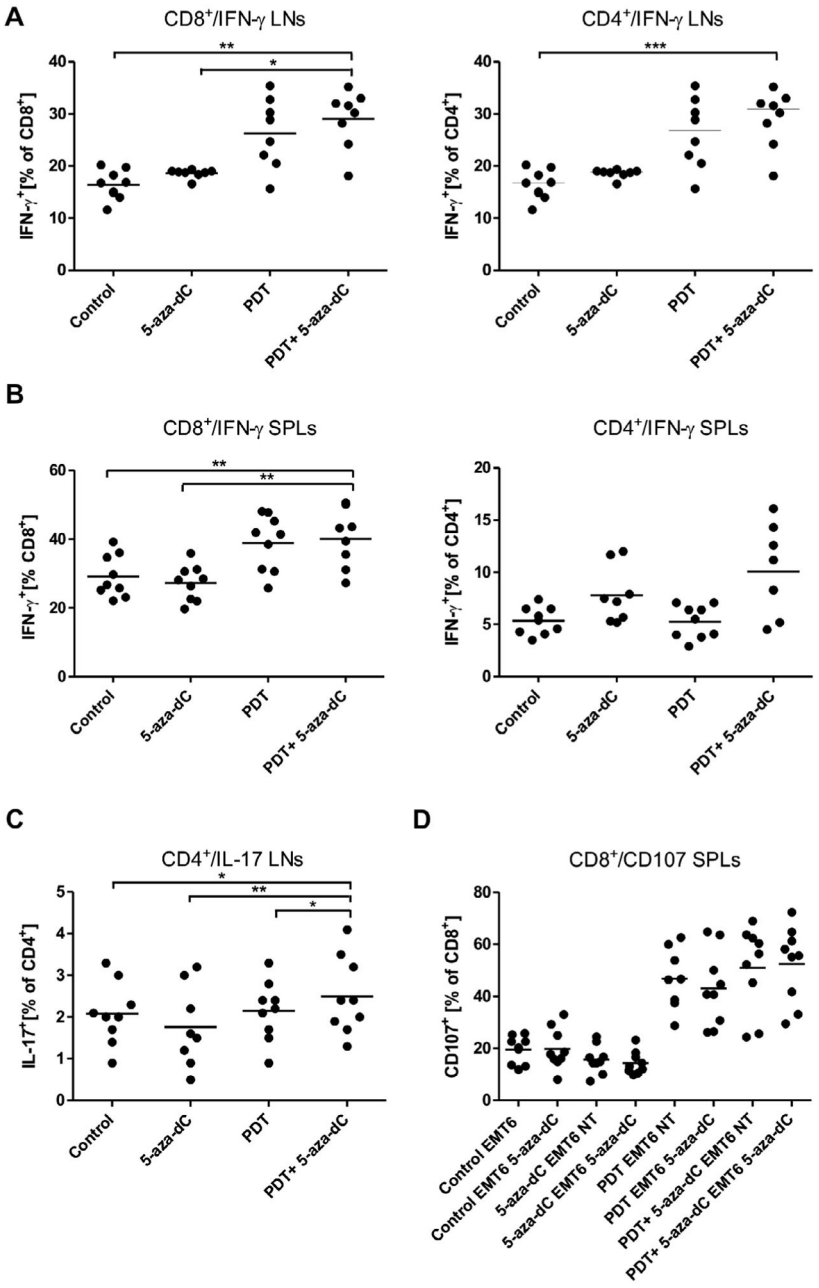

Fig. 5.

Activation of the immune response by treatment combining 5-aza-2′-deoxycitidine (5-aza-dC) with photodynamic therapy (PDT). Experiment was carried out as described and presented in Fig. 4A. Two weeks after PDT tumour bearing mice (n = 8–9) were sacrificed and spleens and tumour draining lymph nodes were harvested. Splenocytes and lymph node (LN) cells were stimulated *ex vivo* for 6 h with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of GolgiPlug. Data show intracellular levels of interferon-γ (IFN-γ) in $CD8^+$ and $CD4^+$ T cells isolated from LNs (A), spleens (B) and interleukin-17 (IL-17) in $CD4^+$ cells obtained from LNs (C) of all experimental groups assessed by flow cytometry

after staining with mAbs (anti-CD4-PerCp-Cy5, anti-CD8- fluorescein isothiocyanate (FITC), anti-IFN-γ-phycoerythrin (PE) and anti-IL-17-allophycocyanin (APC)). 5×10^6 isolated splenocytes were seeded into 48-well plates and co-cultured with 1×10^6 5-aza-dC-treated or non-treated EMT6 target cells. After 3 days of culture cells were re-stimulated for 6 h as described above and surface expression of CD107 was determined on splenocytes co-cultured with EMT6 target cells non-treated (NT) or treated with 10 μM 5-aza-dC after staining with anti-CD8-FITC and anti-CD107-PerCp-Cy7 (D). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ compared with control and single treatment groups (Mann–Whitney test).

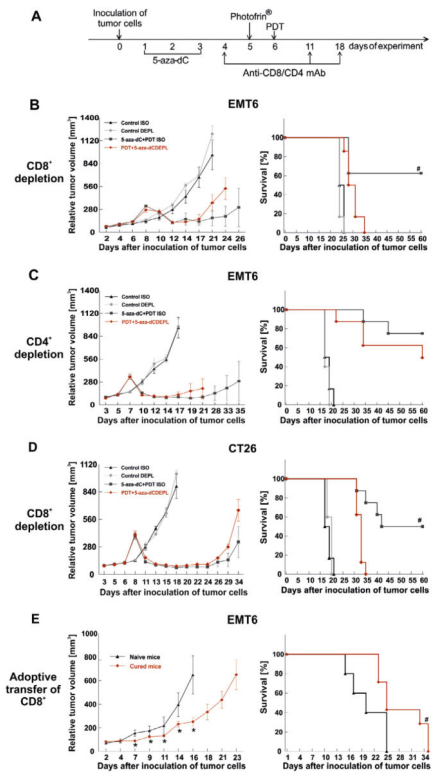

Fig. 6.

5-Aza-2′-deoxycitidine (5-aza-dC) combined with photodynamic therapy (PDT) induces long-term antitumour effect dependent on activation of $CD8^+$ T cells. Graph A presents detailed experimental schedule. Mice (n = 5–8) were treated as previously described. Treatments with anti-CD8, anti-CD4 and matched isotype control mAbs were performed on days 4, 11 and 18 of the experiment. DEPL (depletion) describes a group of mice treated with depleting mAbs and ISO refers to group treated with isotype control mAbs. Left panel shows tumour growth rate right panel shows corresponding Kaplan–Meyer survival plots for depletion of $CD8^+$ T cells in EMT6 (B) and in CT26 (D) tumour model of control and combination therapy groups, depletion of $CD4^+$ T cells in EMT6 (C) model in control and treated mice and $CD8^+$ T cell adoptive transfer from cured and naïve mice in EMT6 tumour model (E). The $CD8^+$ T cells (7×10^6/mouse) isolated from spleens and lymph nodes (LNs) of naïve and cured mice were adoptively transferred i.v. into EMT6 tumour-bearing mice pre-treated with 5-aza-dC. $^*p < 0.05$, compared with all other groups (Student's t test). $^\#p < 0.05$, compared with all other groups (log-rank test).