Combining Type I Interferons and 5-Aza-2′-Deoxycytidine to Improve Anti-Tumor Response against Melanoma

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Resistance to IFN-I–induced antineoplastic effects has been reported in many tumors and arises, in part, from epigenetic silencing of IFN-stimulated genes by DNA methylation. We hypothesized that restoration of IFN-stimulated genes by co-administration of the demethylating drug 5-aza-2′-deoxycytidine (decitabine [DAC]) may enhance the susceptibility to IFN-I–mediated antitumoral effects in melanoma. We show that combined administration of IFN-I and DAC significantly inhibits the growth of murine and human melanoma cells, both in vitro and in vivo. Compared with controls, DAC/IFN-I–treated melanoma cells exhibited reduced cell growth, augmented apoptosis, and diminished migration. Moreover, IFN-I and DAC synergized to suppress the growth of three-dimensional human melanoma spheroids, altering tumor architecture. These direct antitumor effects correlated with induction of the IFN-stimulated gene Mx1. In vivo, DAC/IFN-I significantly reduced melanoma growth via stimulation of adaptive immunity, promoting tumor-infiltrating CD8+ T cells while inhibiting the homing of imnosuppressive CD11b+ myeloid cells and regulatory T cells. Accordingly, exposure of human melanoma cells to DAC/IFN-I induced the recruitment of immune cells toward the tumor in a Matrigel (Corning Life Sciences, Kennebunkport, ME)-based microfluidic device. Our findings underscore a beneficial effect of DAC plus IFN-I combined treatment against melanoma through both direct and immune-mediated anti-tumor effects.

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INTRODUCTION

DNA methylation on cytosine residues is a critical mechanism of epigenetic regulation of gene expression that plays an important role in melanoma progression and metastasis by affecting key cellular events such as cell cycle, DNA repair, apoptosis, invasion, and immune recognition (Fratta et al., 2013; Lee et al., 2014; Timp and Feinberg, 2013). The demethylating agent 5-aza-2′-deoxycytidine (decitabine [DAC]) is an inhibitor of the DNA methyl transferase enzymes.

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Abbreviations: DAC, decitabine; ISG, interferon-stimulated gene; MDSC, myeloid-derived suppressor cell; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; OVA, ovalbumin; PBMC, peripheral blood mononuclear cell; Treg, regulatory T cell

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IFN-stimulated gene (ISG) Mx1 (Zitvogel et al., 2015). In melanoma patients, high intratumoral levels of IFN-I correlate with increased tumor-infiltrating cytotoxic CD8+ T cells and natural killer cells and a favorable prognosis (Wenzel et al., 2005). Indeed, IFN-α is the only approved drug for the adjuvant therapy of advanced melanoma, although its effectiveness may be limited by epigenetic silencing through DNA methylation of ISGs that mediate most IFN-I–mediated antineoplastic actions.

We explored whether DAC could increase melanoma susceptibility to antitumoral effects of IFN-I. We show that the combined administration of IFN-I and DAC significantly inhibits human and murine melanoma cell growth in vitro and in vivo, both through direct effects on melanoma proliferation, cell death, and migration and by recruiting immune effectors toward the tumor.

RESULTS

DAC plus IFN-I promotes cell death and reduces proliferation and migration of B16 melanoma cells

We hypothesized that DAC-induced epigenetic restoration of ISG may enhance the sensitivity of melanoma cells to IFN-I. One single treatment with DAC plus IFN-I caused in B16.F10 melanoma cells long-lasting DNA demethylation (72 hours) and up-regulation of the ISG Mx1 at significantly higher levels, compared with those receiving IFN-I alone, indicating an enhanced and prolonged IFN-I–stimulated response after demethylation (see Supplementary Figure S1a and c online). In a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium (MTS) metabolic assay we found considerable suppression of melanoma cell growth after DAC/IFN-I treatment compared with single treatments, particularly after 72 and 96 hours from treatment when cell viability was significantly suppressed (<10% cell viability) with respect to controls (Figure 1a, and see Supplementary Figure S1b). Treatment with DAC/IFN-I induced significant apoptosis in B16 cells compared with single-treated or untreated controls, visible from 48–96 hours after treatment by annexin V-PI staining (Figure 1b and c) and cell cycle arrest in the G2/M phase (Figure 1d). Thus, DAC augments IFN-I–induced suppression of melanoma growth in vitro, partly through stimulation of apoptotic cell death and by blocking cell cycle. We next determined whether DAC/IFN-I could affect the migratory ability of melanoma cells. In a wound-healing scratch assay, untreated B16 cells easily migrated into an open space (scratch in the confluent cell layer) and filled it within 24 hours. In contrast, melanoma cells treated with DAC or IFN-I left a wound margin between the two scratched edges (Figure 1e). Of note, the margin left from DAC/IFN-treated B16 cells was significantly wider than those observed for single treatments (Figure 1e and f). Thus, DAC plus IFN-I effectively inhibits melanoma migration and motility.

Combined DAC/IFN-I restricts melanoma growth in vivo by altering the immune microenvironment

To evaluate whether DAC/IFN-I treatment could affect melanoma cell growth in vivo a Matrigel (Corning Life Sciences, Kennebunkport, ME) plug assay was used. Hematoxylin and eosin staining of plug sections at day 10 showed high tumor cell density and absence of necrotic areas in untreated and single-treated B16 plugs (see Supplementary Figure S2a online). In marked contrast, DAC/IFN-I–treated B16-plugs displayed wide empty areas devoid of outgrowing tumor and increased immune infiltrates, as shown by immunofluorescence staining and by flow cytometry analysis of CD45+ leukocytes (see Supplementary Figure 2 b and c). Thus, DAC plus IFN-I effectively reduces melanoma growth in vivo and promotes immune cell recruitment. Next, we assessed the therapeutic efficacy of DAC plus IFN-I in vivo in mice receiving subcutaneous transplants with B16.F10 melanoma. One intraperitoneal injection of DAC plus five daily intratumoral administrations of IFN-I significantly delayed tumor growth (Figure 2a) and increased the survival rate (45% at day 40, Figure 2b) in tumor-bearing mice compared with single-treated or phosphate buffered saline-treated groups. Melanoma tissue sections showed absence of necrotic areas or tumor infiltrates in DAC-treated and control mice, whereas tumors from IFN-I–treated mice showed some infiltrates and limited areas of tumor necrosis (Figure 2c). Wide necrotic areas and large amounts of immune (CD45+) infiltrates were found in melanoma tissues from DAC/IFN-I–treated mice (Figure 2c and d). Flow cytometry analysis of tumor-infiltrating CD45+ populations evidenced an increase in CD8+ T-lymphocyte frequencies and diminished Tregs and CD11b+ myeloid cells, particularly Ly6C+Ly6G+ granulocytic MDSCs (Figure 2e) in mice receiving DAC/IFN-I therapy. The increased levels of tumor-infiltrating CD8 T cells correlated with up-regulation of CD8 T cell-attracting chemokines CCL2, CCL3, CCL5, and T helper type 1-related CXCL9, CXCL10, IL-12, and Granzyme B (Figure 2f) (Harlin et al., 2009). In addition, the low frequencies of MDSCs and Tregs matched with marked down-modulation of intratumoral GM-CSF and CXCL12 (Figure 2e) (Bronte et al., 1999; Obermajer et al., 2011; Zou et al., 2004). In vitro transwell migration assay of MDSC toward melanoma cells confirmed that DAC/IFN-I acted by modulating intratumoral release of MDSC-attracting chemokines (see Supplementary Figure S3d online). Mx1 was also significantly up-regulated in these tumors, indicating that selective expression of ISGs could be efficiently induced by this combined treatment in vivo (Figure 2g).

The therapeutic efficacy of DAC/IFN-I in vivo required adaptive anti-tumor immunity, because this treatment failed to restrain melanoma growth in severe combined immunodeficiency mice, which lack functional B and T lymphocytes (Figure 3a). To evaluate the systemic antitumoral effects of the combined treatment, we performed transplantations in mice with ovalbumin (OVA)-expressing B16 melanoma cells. Flow cytometry analysis of immune spleen cell populations in B16.OVA-bearing mice undergoing DAC/IFN-I therapy showed a moderate, but significant, increase in CD8 T-cell frequencies (Figure 3b) and increased proliferative response to major histocompatibility complex-I–restricted OVA peptide (SIINFEKL) or OVA protein with respect to controls (Figure 3c). In addition, in a bilateral tumor model in which only one melanoma nodule received intratumoral IFN-I, DAC/IFN-I treatment significantly reduced the growth of the contralateral (not treated) tumor with respect to control and DAC-treated mice, albeit not to IFN-I–treated mice (Figure 3d).
Because IFN-I has been shown to up-regulate the expression of the checkpoint inhibitors PD-1 and PDL-1 (Kakizaki et al., 2015), we evaluated whether DAC could increase these IFN-I-induced effects. With respect to IFN-I alone, DAC/IFN-I markedly increased melanoma cell expression of PDL-1 in vivo and in vitro (Figure 3 e) but not PD-1 expression on tumor-infiltrating CD8 T cells (Figure 3 e). Overall, these data indicate that DAC plus IFN-I delays melanoma growth in vivo by creating an immune microenvironment more favorable for tumor control, linked to amplification of IFN-I signaling, and suggest that this regimen may be further improved by combining immunotherapy targeting the PD-1/PDL-1 axis to boost systemic antitumor immunity.

DAC plus IFN-I exhibits antitumor effects against human melanoma cells and three-dimensional spheroids

Combined exposure to DAC plus IFN-I (IFN-α2) effectively suppressed the growth of human melanoma cells, as evidenced by a significant decrease in cell viability relative to untreated and single-treated conditions at 48 hours (70%), 72 hours (40%), and 96 hours in A375 cells (50%) (Figure 4a, and see Supplementary Figure S4a and c online) and in SK-MEL-28 (58%), SC (55%), and WM793 cells (40%) (Figure 4b). Moreover, exposure of human melanoma cells to DAC/IFN-I resulted in increased levels of apoptosis (Figure 4c and d) and S-phase or G2/M-phase cell cycle arrest (see Supplementary Figure S4e) compared with single treatments or untreated controls. Furthermore, DAC/IFN-I also inhibited the migratory ability of human melanoma cells, as shown in a wound-healing scratch assay (Figure 4e and f) and in a transwell migration assay (see Supplementary Figure S4d).

We next used three-dimensional human melanoma spheroids as a more representative model for drug efficacy than two-dimensional cell culture systems, mimicking the complex and heterogeneous architecture of the tumor tissue in vivo (Zanoni et al., 2016). Early-formed A375 melanoma spheroids (mean Feret diameter = 200–250 μm) were treated once with DAC, IFN-I, or both and their growth was monitored. Untreated and single-treated spheroid units grew progressively with time, reaching a mean Feret diameter of 600 μm by 5 days of culture. In contrast, DAC/IFN-I effectively synergized to block the expansion of melanoma spheroids over 5 days (Figure 5a and b). This effect was paralleled by significant up-regulation of Mx1 expression selectivity in three-dimensional spheroids exposed to DAC/IFN-I (Figure 5c). Of interest, DAC/IFN-I were also effective in contrasting tumor spheroid formation, when administered at the time of A375 cell seeding, as evidenced by highly disorganized tumor architecture and reduced tumor diameter.
(158 μm), compared with untreated (240 μm), DAC (200 μm), or IFN-I (254 μm) conditions after 10 days (see Supplementary Figure S5 online). These data suggest that DAC/IFN-I effectively suppresses human melanoma growth in both two- and three-dimensional culture systems.

**DAC/IFN-I treatment of human melanoma cells stimulates immune cell recruitment**

To address whether DAC/IFN-I could trigger immune cell recruitment in human melanoma cells, as seen in the murine model, we used an organ-on-chip approach, a microfluidics-based technology recently developed in our laboratories as a reliable tool to measure tumor-immune cell interactions (Businaro et al., 2013; Vacchelli et al., 2015). An ad hoc fabricated device, composed of three main fluidic chambers and two narrow gel-containing chambers, interconnected by two arrays of microchannels, was used to co-culture human peripheral blood mononuclear cells (PBMCs) with human melanoma cells, allowing the comparison of two different treatment conditions of melanoma cells simultaneously (see Supplementary Figure S6 online). Thus, human A375 cells labeled with PKH67 green fluorescent dye were resuspended in Matrigel in the presence or absence of DAC and/or IFN-I and were loaded into each narrow chamber to embed the tumor cells in a gel matrix, whereas PKH26 red-labeled PBMCs were loaded into the central fluidic chamber (see Supplementary Figures S6 and S7 online). When DAC/IFN-treated A375 melanoma cells were co-loaded with the untreated melanoma cells and confronted for their capacity to attract PBMCs, displacement of PBMCs toward the right microchannel array interconnecting to treated-A375 melanoma cells was evident at 24 hours (right chamber) (see Supplementary Figure S8 online). At 48 hours, PBMCs visibly migrated and infiltrated the right-side microchannels, whereas no migration was observable toward the left microchannel array connecting to untreated A375 cells (left chamber) (see Supplementary Figure S9 and Supplementary Videos S1 and S2 online). After 72 hours, massive infiltration of PBMCs could be observed in the gel matrix containing A375-DAC/IFN cells (right), whereas rare PBMCs were found in the gel containing untreated A375 cells (left) (Figure 6a). When DAC/IFN-treated A375 melanoma cells were confronted with DAC-treated (Figure 6b) or IFN-treated (Figure 6c) melanoma cells, again, preferential homing of
PBMCs toward the gel matrix containing DAC/IFN-treated melanoma gel chamber could be observed at 72 hours (Figure 6b–d). Similar results were obtained with SC, SK-MEL-28, and WM793 human melanoma cell lines (Figure 6e, and see Supplementary Figure S10 and Supplementary Videos S3 and S4 online). Thus, these findings strongly indicate that DAC/IFN-I treatment of human melanoma cells selectively induces potent attractive signals that recruit immune cells.

**DISCUSSION**

High-dose IFN-α2b is the only currently available adjuvant therapeutic option for advanced melanoma after resection (Kaufman et al., 2015). However, the degree to which IFN-I offers real clinical benefits is challenged by multiple escaping mechanisms operated by the tumor (Chevolet et al., 2015; Lee et al., 2014; Umansky and Sevko, 2012). Melanoma progression is associated with epigenetic alterations, such as aberrant methylation of DNA by DNMT enzymes, that result in silencing of genes encoding for cancer antigens, oncosuppressors, or immune-activating signals. Therefore, epigenetic modifier drugs, such as DNA methyl transferase inhibitors, have gained much interest in virtue of their high potential for improving the efficacy of immunotherapies (Saleh et al., 2016).

In this study, we explored the effects of combining the DNA demethylating agent DAC with IFN-I as a strategy for overcoming melanoma resistance to IFN-I. One single
administration of low-dose DAC plus IFN-I resulted in multiple antitumor effects against murine and human metastatic melanoma cells. First, the combined treatment induced significant cell growth inhibition visible up to 4 days after drug exposure that accounted partly for induction of apoptosis and partly for G2/M–S-phase cell cycle arrest.
similar to a previous study using repeated administrations of DAC (over 4 consecutive days) followed by IFN-α2b (Reu et al., 2006). In addition, DAC/IFN-I also inhibited the migration/motility of melanoma cells, in accordance with previous reports showing reduced migration and motility in melanoma cells overexpressing IFN-α (Rossi et al., 2015).

DAC/IFN-I effectively suppressed both the formation and expansion of three-dimensional human melanoma spheroids in a synergistic manner. These antitumoral effects correlated with a substantial increase in Mx1 expression levels in DAC/IFN-I-treated melanoma cells and three-dimensional spheroids compared with melanoma cells treated with IFN-I alone, indicating that DAC enhanced the response to IFN-I. The up-regulation of Mx1 expression in DAC/IFN-I-treated melanoma cells correlated with DAC-induced DNA demethylation, suggesting reactivation of ISGs induced by DNA demethylation. Accordingly, demethylation of ISGs in melanoma and other tumor cell lines by DNA methyl transferase enzymes, including DAC, has been widely reported, and a mechanism involving endogenous retrovirus activation by DNA methyl transferase enzymes leading to IFN-I response has emerged (Chiappinelli et al., 2015; Karpf et al., 1999; Reu et al., 2006). In our model, IFN-I was able to induce toll-like receptor 3 and MDA5 expression far more efficiently (up to 40- and 80-fold, respectively; see Supplementary Figure S1d) than DAC, suggesting that the response induced by IFN-I itself in melanoma cells likely prevailed over that possibly induced by DAC-stimulated endogenous retrovirus. It is possible, however, that IFN-mediated induction of toll-like receptor 3 and MDA5 signaling may amplify double-stranded RNA stimulation after endogenous retrovirus activation by DAC, as proposed elsewhere (Kang et al., 2002; Siren et al., 2005; Tissari et al., 2005). In this regard, it is worth mentioning that IFN-I itself can promote the reactivation of silenced ISGs in cancer cells by inducing reversible DNA demethylation in the promoter region (Micali et al., 2007), further suggesting a possible cooperation between DAC and IFN-I in maintaining DNA in a demethylated status in melanoma cells.

The anti-tumor effects of DAC/IFN-I in vivo denoted also an indirect action of these drugs, through recruitment of immune cells. In our model, IFN-I was able to induce toll-like receptor 3 and MDA5 expression far more efficiently (up to 40- and 80-fold, respectively; see Supplementary Figure S1d) than DAC, suggesting that the response induced by IFN-I itself in melanoma cells likely prevailed over that possibly induced by DAC-stimulated endogenous retrovirus. It is possible, however, that IFN-mediated induction of toll-like receptor 3 and MDA5 signaling may amplify double-stranded RNA stimulation after endogenous retrovirus activation by DAC, as proposed elsewhere (Kang et al., 2002; Siren et al., 2005; Tissari et al., 2005). In this regard, it is worth mentioning that IFN-I itself can promote the reactivation of silenced ISGs in cancer cells by inducing reversible DNA demethylation in the promoter region (Micali et al., 2007), further suggesting a possible cooperation between DAC and IFN-I in maintaining DNA in a demethylated status in melanoma cells.

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**Figure 5.** DAC and IFN-I synergize to halt human melanoma three-dimensional spheroid expansion. (a) Multicellular tumor spheroids (200–250 μm in size) from A375 human melanoma cells received DAC (0.25 μmol/L) and/or IFN-α2 (500 U/ml). Phase-contrast micrographs were obtained at the indicated times after treatment. Scale bar = 200 μm. (b) Quantitative determination of tumor spheroids growth. Data show the mean Feret diameter ± standard deviation at the depicted times (n = 6). (c) Expression of Mx1 by quantitative PCR in spheroids at day 5 after treatment. *P < 0.05, **P < 0.01, ***P < 0.001. DAC, decitabine; NT, not treated.

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effectors. In a Matrigel plug model, addition of DAC/IFN-I to the B16 melanoma-gel mixture elicited substantial recruitment of CD45<sup>+</sup> cells. Furthermore, tumor growth reduction in mice implanted with B16 melanoma after systemic DAC (intraperitoneal) plus local IFN-I (intratumorally) combined therapy delineated an active recruitment of immune infiltrates surrounding vast tumor necrotic areas. Induction of immune cell recruitment after DAC/IFN-I treatment could also be shown in human melanoma cells by means of an organ-on-chip approach, a microfluidics-based technology recently described in our laboratory as a reliable in vivo-like system for investigating the crosstalk between immune cells and cancerous cells (Boussommier-Calleja et al., 2016; Businaro et al., 2013; Mattei et al., 2014; Vacchelli et al., 2015). By confronting two different treatment conditions of human melanoma cells embedded in Matrigel for their ability to attract PBMCs, we could show clear preferential migration of these immune cells toward the DAC/IFN-I–exposed melanoma cells with respect to control cells. Our analysis accounted for PBMCs actively migrating within the gel matrix enclosing the melanoma cells, thus closely mimicking the tumor microenvironment.

Local administration of IFN-I in combination with chemotherapy or immunotherapy has proven an effective strategy not only to limit the risks of adverse effects linked to its systemic use but also to boost antitumor responses (Dubrot et al., 2011; Schiavoni et al., 2011). Intratumoral IFN-β can modulate the tumor microenvironment by reducing the suppressive activity of MDSCs, Tregs, and tumor-associated macrophages and inducing dendritic cell activation, leading to antitumor immunity (Kakizaki et al., 2015; Van der Jeught et al., 2014). In melanoma patients, peritumoral injection of IFN-β induces the recruitment of tumor-reactive CD8<sup>+</sup> T cells into the tumor microenvironment (Fujimura et al., 2009; Lim et al., 2014). Furthermore, intratumoral induction of IFN-I was shown to be crucial for the antitumor efficacy of

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**Figure 6.** PBMCs preferentially migrate toward DAC/IFN-I–treated human melanoma cells in a microfluidic device. PKH26-labeled (red) PBMCs were loaded in the central chamber of microfluidic devices. PKH67-labeled (green) human melanoma cells embedded in Matrigel (Corning Life Sciences, Kennebunkport, ME) containing treatments were placed in lateral chambers. (a) Untreated A375 cells (NT, left channel) versus A375 plus DAC/IFN (right channel). (b) A375 cells plus DAC (left) versus A375 plus DAC/IFN (right). (c) A375 cells plus IFN-I (left) versus A375 plus DAC/IFN (right). Fluorescence images were obtained after 72 hours of culture. Discontinued vertical white lines depict microchannel area. Scale bars = 100 μm. (d) Quantitative analysis of PBMC infiltration in DAC/IFN. A375 melanoma channels calculated as red fluorescence values in right/left channels at the indicated times. (e) Quantitative analysis of preferential PBMC infiltration toward DAC/IFN-treated (vs. untreated) SK, SC, and WM793 melanoma cell-containing channels at the indicated times. *P < 0.05, ***P < 0.001 versus 24 hours. NT, not treated; PBMC, peripheral blood mononuclear cell; SK, SK-MEL-28.
radiotherapy because it mediated the recruitment and cytolytic activity of CD8 T cells (Burnette et al., 2011). Here, DAC/IFN therapy induced substantial increase, versus IFN-I alone, in tumor-infiltrating CD8+ T lymphocytes with concomitant reduction of immunosuppressive CD11b+ myeloid cells, especially Ly6ClowLy6G+ granulocytic MDSC, and Tregs through modulation of chemokine/cytokine expression at the tumor site. This assumption is supported by our in vitro assays with MDSCs showing that DAC/IFN-I acted by impairing MDSC homing toward melanoma cells, rather than affecting MDSC life span or differentiation (see Supplementary Figure S3). In patients with advanced melanoma, elevated frequencies of total and granulocytic MDSCs indicate poor prognosis, and the clinical effects of IFN-α–based therapies were shown to correlate with reduction of these populations (Jiang et al., 2015; Tarhini et al., 2012). Thus, the immune profile observed selectivity in mice treated with DAC/IFN-I is a common trait of antitumor responses and strongly correlates with tumor growth reduction.

Although DAC/IFN-I induced activation of systemic immune responses, it only partly controlled the growth of distant tumors. These findings indicate that the beneficial additive effects of DAC/IFN-I therapy occurred principally at the local level, through remodeling of the tumor microenvironment. In this regard, the finding that DAC/IFN-I, with respect to IFN-I alone, both enhances tumor cell expression of PDL-1 and increases tumor-infiltrating CD8 T cells suggest that this regimen may represent a promising platform to increase the therapeutic response to immunotherapies blocking the PD-1/PDL-1 axis (Gajewski et al., 2010; Hamid et al., 2011; Ji et al., 2012). In this regard, a wide range of treatment combinations for multimodal therapy with immune-checkpoint blockade-based immunotherapy have been explored (Zamarin and Postow, 2015). Encouraging results from combinations with CTLA4 or PD1/PDL1 inhibitors have previously described (Tovey et al., 1974). A375, WM793, SK-MEL-28, and SC human melanoma cells were treated once with DAC and/or human IFN-α2b (Merck, Sharp & Dohme Limited, Hoddesdon, UK). The drug doses for each cell line were chosen on the basis of dose-response experiments in MTS cell viability assay and are shown in Supplementary Table S1 online. Primers used for reverse transcription-PCR are shown in Supplementary Table S2 online.

Mice and in vivo treatments
Subconfluent B16.F10 or B16.OVA melanoma cells were injected subcutaneously in 6-week-old female C57BL/6 or severe combined immunodeficiency mice. Mice were injected once intraperitoneally with DAC (1 mg/kg) and/or intratumorally with murine IFN-I (5 x 10^6 U). IFN-I injections were repeated for 4 consecutive days for a total of five administrations. All animal procedures were performed according to Italian and European regulations and were approved by the Ethical Committee for Animal Experimentation of the Istituto Superiore di Sanità (Rome, Italy).

Statistical analysis
One-way analysis of variance was performed to compare means among groups, followed by post hoc testing (Tukey). Log-rank Mantel-Cox test was used for the analysis of survival curves. Values were considered as significant when the probability was below the 5% confidence level (P < 0.05).

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.08.024.

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