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Upregulation of PD-L1 expression by resveratrol and piceatannol in breast and colorectal cancer cells occurs via HDAC3/p300-mediated NF-κB signaling

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Abstract. Programmed cell death ligand 1 (PD-L1) expressed in cancer cells interacting with its receptor programmed cell death 1 (PD-1) expressed in immune cells represents a regulatory axis linked to the suppression and evasion of host immune functions. The blockade of PD-1/PD-L1 interaction using monoclonal antibodies has emerged as an effective therapy for several solid tumors; however, durable response has been observed in a subset of patients with PD-L1-positive tumors. Thus, the understanding of the mechanisms responsible for the expression of PD-L1 in tumor cells may help to improve the response to PD-L1 blockade therapies. In this study, we investigated whether resveratrol, a grape-derived stilbenoid with immunoregulatory activity, modulates the expression of PD-L1 in breast and colorectal cancer cells. The surface expression of PD-L1 was determined by flow cytometry in cancer cells treated with resveratrol and/or piceatannol. Each stilbenoid alone induced PD-L1 and when used in combination, elicited a synergistic upregulation of PD-L1 in some cell lines. The induction of PD-L1 by the combined use of stilbenoids was most pronounced in the Cal51 triple-negative breast cancer (TNBC) and SW620 colon cancer cells. The observed induction of PD-L1 was transcriptionally mediated by nuclear factor (NF)-κB, as shown by NF-κB reporter assays, the nuclear accumulation of the p65 subunit of NF-κB, inhibition by the IKK inhibitor, BMS-345541, and histone the modification inhibitors, resminostat, entinostat or anacardic acid. Combined treatment with resveratrol and piceatannol also decreased tumor cell survival as indicated by the upregulation of the DNA damaging marker, γH2AX, the cleavage of caspase 3, the downregulation of the survival markers, p38-MAPK/c-Myc, and G_1-to-S cell cycle arrest.

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Introduction

Programmed cell death ligand 1 (PD-L1) is a functional ligand of programmed cell death 1 (PD-1) (1). The binding of tumor cell PD-L1 to immune T-cell PD-1 inhibits T-cell activation and attenuates T-cell-mediated immunosuppression (2-4). This results in the evasion of host antitumor immunity, potentially reducing the efficacy of anticancer therapies and resulting in a poor clinical outcome (2,5,6). To counter the escape from the host immune surveillance system by tumor cells, blockade strategies using monoclonal antibodies (mAbs) to prevent the binding of PD-L1 to PD-1 have been developed (7-12). The clinical efficacy of this approach has been demonstrated in certain cancer types, including melanoma (13-16), non-small-cell lung cancer (17-19) and renal carcinoma (20). Blockade therapy differs from tumoricidal chemotherapy in that its antitumorigenic effects involve boosting host immunity concomitant with the modulation of the expression/activity of the repertoire of cytotoxic T-cell and T-regulatory cells (21-24). Although success in anti-PD-1/PD-L1 therapy has made it possible to achieve tumor eradication and disease remission/cure, outstanding challenges remain. Only 31% of patients with advanced melanoma treated intravenously with anti-PD-1 drugs (nivolumab) have exhibited objective tumor regression (14). Likewise, the same therapeutic regimen has shown response rates ranging from 25% in renal cancer (20), to 19% in non-squamous non-small-cell lung cancer (17) and to 20% in squamous non-small-cell lung cancer (18), respectively. Moreover, a positive therapeutic response typically occurs in patients with PD-L1-positive tumors (13,25).

Since the expression of PD-L1 in cancer cells may affect the patient response to immune blockade therapy, it is of interest to identify agents capable of modulating the expression of PD-L1. In this study, we focused on resveratrol, a stilbenoid present abundantly in red wine, red grape skin and peanuts (26,27). Interest in resveratrol stems largely from the report in 1997 by Jang *et al*, showing that the molecule prevents the development

of pre-neoplastic lesions in carcinogen-exposed mammary glands, and the inhibition of initiation and promotion of skin cancer in a mouse model (28). Since then, numerous studies have demonstrated its broad-spectrum beneficial health effects, including, anti-inflammatory (29,30) and anticancer activities (26). Several mechanisms (31,32) and target proteins for the biological and pharmacological activities of resveratrol have been identified and characterized (33-42). Resveratrol has also been reported to exert immunomodulatory effects, as illustrated by the induction of interferon (IFN)-γ expression in $CD8^+$ T-cells both *ex vivo* and *in vivo* (43), and the inhibition of the proliferation of $CD4^+$ T-cells (43,44). Craveiro *et al* (45) recently demonstrated that low-dose resveratrol (20 μM) activates human $CD4^+$ cells and induces DNA damage response, while high-dose resveratrol (100 μM) induces G_1 phase cell cycle arrest, suggesting that resveratrol may act on host immune cell types in a dose-dependent manner. The chemopreventive activity of resveratrol was first demonstrated using skin and breast cancer models (28), and recent clinical trials support the use of resveratrol in colorectal cancer (46,47). Thus, in this study, we selected breast and colorectal cancer cell lines to examine the regulatory effects of resveratrol and its biotransformed product, piceatannol, on the expression of PD-L1. The results revealed that both dietary stilbenoids, alone or in combination, copiously increased the expression level of PD-L1 in some breast and colorectal cancer cells via HDAC3/p300-mediated nuclear factor (NF)-κB signaling. In addition, both stilbenoids exerted cytotoxic effects on the tumor cells.

Materials and methods

Reagents. Resveratrol, piceatannol, resminostat, entinostat, mocetinostat, vorinostat, curcumin, garcinol, anacardic acid and Tip60i were purchased from Selleckchem (Houston, TX, USA). MB-3 and BMS-345541 were purchased from MilliporeSigma (Burlington, MA, USA). Pterostilbene and myricetin were from LKT Laboratories (St. Paul, MN, USA) and trimethoxy-resveratrol (trans-3,5,4'-trimethoxystilbene) was from Cayman Chemical Co. (Ann Arbor, MI, USA). Stock solutions of the chemicals were prepared based on the information provided by the manufacturer and maintained at -20˚C. The antibodies for human PD-L1 (E1L3N, 13684), p38 MAPK (D13E1, 8690), NF-κB p65 (D14E12, 8242), γH2AX (20E3, 9718), cleaved caspase 3 (D3E9, 9579), IRF-1 (D5E4, 8478) and rabbit IgG isotype monoclonal antibody (DA1E, 5742) conjugated to PE were obtained from Cell Signaling Technology (Beverly, MA, USA). c-Myc antibody (9E10, sc-40) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Fetal bovine serum, RPMI-1640, DMEM, streptomycin and penicillin were from Gibco/Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals and solvents were of analytical grade.

Cell culture and treatment. Human BT549 (breast cancer), BT474 (invasive ductal carcinoma), SKBR3 (breast cancer), HCT116 (colon cancer), SW480 (colon cancer), HT29 (recto-sigmoid adenocarcinoma) and SW620 (colon cancer) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Human Cal51 (breast cancer) cells were from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The cells were maintained in RPMI-1640 or DMEM culture media supplemented with penicillin, streptomycin and 10% heat-inactivated fetal bovine serum according to the manufacturer's instructions. The cells were split once a week and the media were changed every 3-4 days.

In all experiments described in this study, parent/parental or DMSO treated cells all refer to untreated, control cells. For treated cells, the conditions (dose and treatment duration) and whether any reagents were used together at specific doses were as indicated in the figure legends.

Immunohistochemistry. Paraffin-embedded SW620 colon cancer cells were immunohistochemically stained to evaluate the protein expression of PD-L1, c-Myc, p38 MAPK, γH2AX and cleaved caspase 3. Following deparaffinization and rehydration, sections of SW620 cells were prepared. The slides were heated in the Retriever 2000 pressure cooker (Electron Microscopy Sciences, Hatfield, PA, USA) in Borg buffer pH 9.5 (Biocare Medical, Concord, CA, USA) and cooled to room temperature. Endogenous peroxidase activity was inactivated with Peroxidazed 1 (Biocare Medical) for 10 min. Non-specific protein interactions were blocked for 10 min with Background Punisher (Biocare Medical). The sections were incubated with the primary antibodies, indicated above, at a dilution of 1/200 for 1 h, washed in TBS and incubated with SignalStain Boost IHC Detection Reagent (Cell Signaling Technology) for 30 min. Following washes in TBS, immunoreactivity was visualized by development with 3,3'-diaminobenzidine (DAB^+, Dako, Carpinteria, CA, USA) for 5 min. Immunostained sections were briefly counterstained with CAT hematoxylin, washed in tap water, dehydrated in a graded alcohol series, cleared in xylene and coverslipped with Permount mounting medium (Fisher Scientific Co. L.L.C., Pittsburgh, PA, USA).

Flow cytometric analysis for the surface expression of PD-L1. The cells were harvested and fixed in 4% paraformaldehyde for 10 min. The cells were then rinsed twice with phosphate-buffered saline (PBS) before a 30-min staining was performed to prepare the samples for flow cytometry using a rabbit anti-human PD-L1 monoclonal antibody (E1L3N) conjugated to phycoerythrin (PE). As controls, cell samples were also stained for 30 min using rabbit IgG isotype monoclonal antibody (DA1E) conjugated to PE. Following labeling with antibody, the cells were rinsed twice with PBS and re-suspended with PBS. The data shown as the geometric means from n=3-4 independent experiments were acquired on a DB LSR Fortessa X-20, and analyzed with FlowJo version 10 software (BD Biosciences, San Jose, CA, USA).

PD-L1 mRNA analyses using the Cancer Cell Line Encyclopedia (CCLE) database. The basal PD-L1 mRNA expression levels shown in Fig. 2B, presented as transcript per million (TPM) were obtained from a public Cancer Cell Line Encyclopedia (CCLE, https://portals.broadinstitute.org/ccle/home) database for cancer cell lines tested.

NF-κB reporter assay. The A549-Dual cells purchased from InvivoGen (San Diego, CA, USA) were used. These are derivatives of the A549 human lung carcinoma cells containing the

stable integration of two inducible reporter constructs. The constructs allow for the co-expression of a secreted embryonic alkaline phosphatase (SEAP) reporter gene under the control of the IFN-β minimal promoter fused 5 five NF-κB binding sites, and, a Lucia luciferase gene encoding a secreted luciferase whose transcription is driven by an ISG54 promoter fused to 5 IFN-stimulated response elements. The cells were treated for the specified amount of time (8, 24 and 48 h) with resveratrol or piceatannol, each at 100 μM or combined, each at 50 μM (referred to as combo-100) to yield a concentration of 100 μM and the secreted alkaline phosphatase and Lucia luciferase in the supernatant of the control and treated cells were detected using the Quanti-Blue reagent from InvivoGen. The results were scored by the fluorescence intensity on a Perkin Elmer EnSpire set at a wavelength of 650 nm (PerkinElmer Inc., Shelton, CT, USA). To determine the role of NF-κB in mediating the induction of PD-L1, the IKK inhibitor, BMS-345541, was administered for 24 h *in vitro* prior to exposure to the combination of piceatannol and resveratrol, each at 50 μM to yield a concentration of 100 μM, for a further 48 h. BMS-345541 was added at increasing concentrations (1, 4 and 8 μM), while the dose of the combination was kept constant.

Cell cycle/apoptosis analysis. The cells were harvested and washed with PBS then re-suspended in cold 1% formaldehyde in PBS solution for 15 min at 4˚C. The cells were washed twice in PBS and re-suspended in ice-cold 70% ethanol and stored at -20˚C for 2 h prior to analysis. Prior to fluorescence measurement the cells were stained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI). The intensity of blue fluorescence emission of DAPI stained DNA, excited with the UV laser (355 nm) was measured, recorded and analyzed on a MoFlo flow cytometer (Beckman Coulter Life Sciences, Indianapolis IN, USA) using Kaluza fluorescence intensity analysis software (48). Experiments were repeated and representative data are presented.

Statistical analysis. All data are presented as the mean ± the standard error of the mean. A Student's t-test or two-way ANOVA with Bonferroni correction were performed to determine statistical significance between frequencies or mean fluorescence intensities of assessed cell populations using GraphPad Prism 6 (GraphPad Software). Statistical significance of results was as indicated in each figure.

Results

PD-L1 expression is increased by dietary stilbenoids, resveratrol and/or piceatannol in breast and colorectal cancer cells. We first assessed any alterations in PD-L1 levels using the Cal51 breast cancer and HCT116 colon cancer cells treated with stilbenoids, specifically, resveratrol, piceatannol, pterostilbene and 3,5,4'-trimethoxystilbene, for comparison with the flavonoid, myricetin (the chemical structures of the compounds are shown in Fig. 1A). The surface expression of PD-L1 in the control (DMSO-treated, also referred to as parent/parental cells) and treated Cal51 and HCT116 cells was assayed by flow cytometry. Resveratrol significantly increased the expression of PD-L1 in the Cal51 cells, while treatment with piceatannol resulted in a marked increase in the PD-L1 level in HCT116 cells (Fig. 1B).

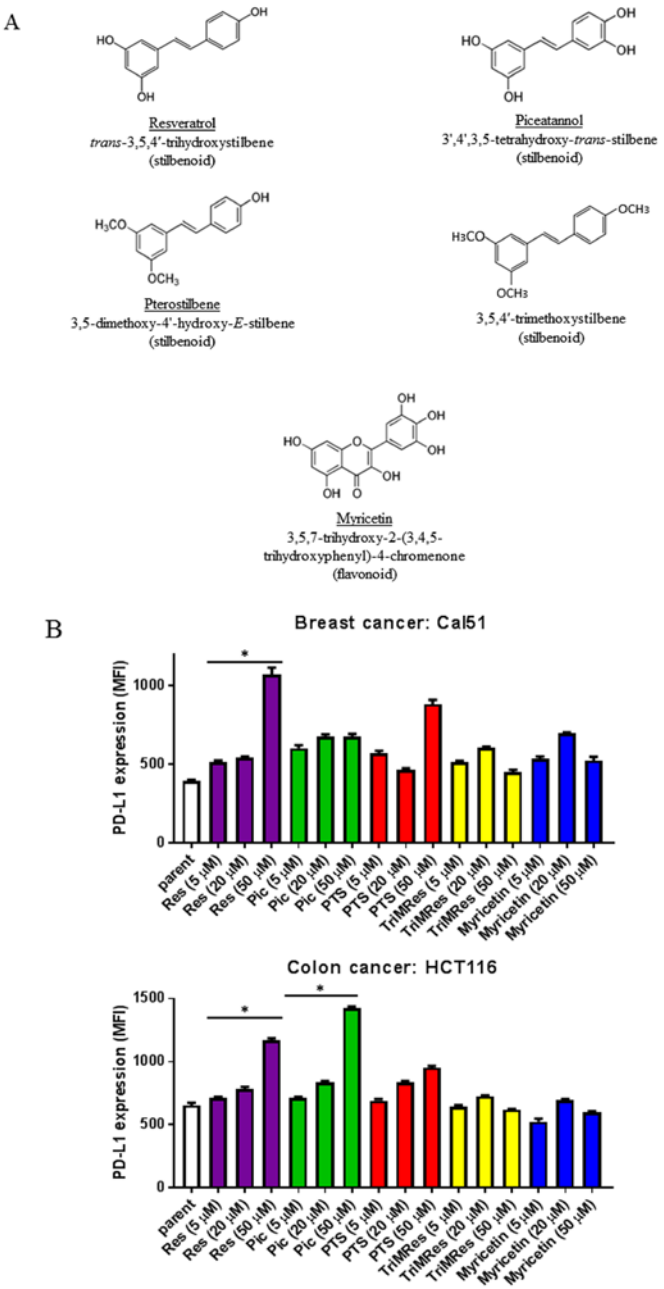
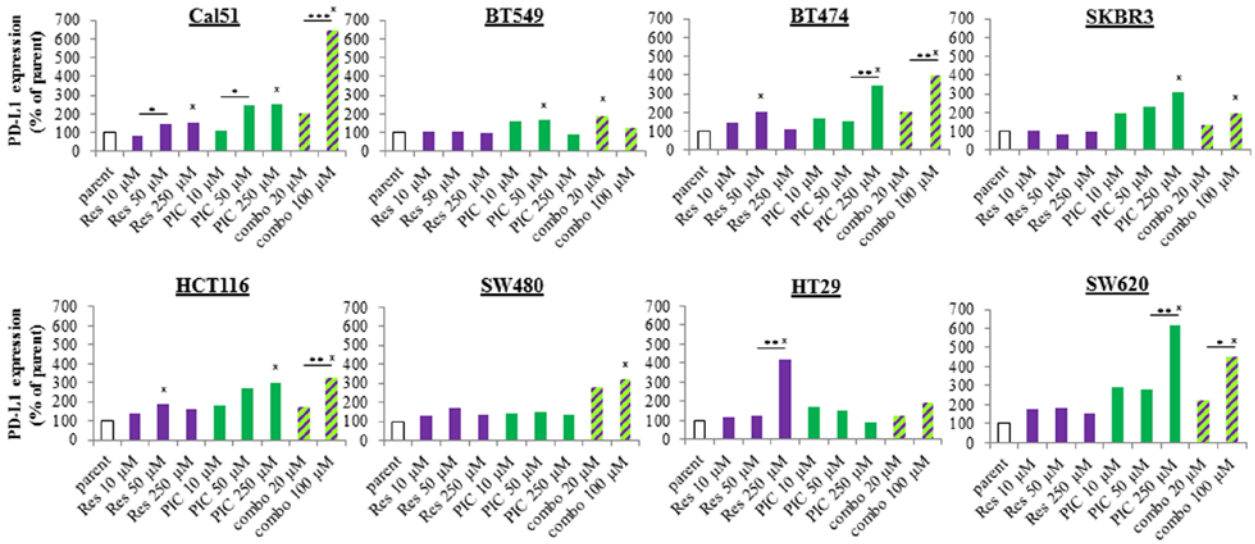


Figure 1. Effects of dietary polyphenols on programmed cell death ligand 1 (PD-L1) expression in human breast and colon cancer cells. (A) Chemical structure of each of the polyphenols tested. (B) The Cal51 human breast cancer cell line and HCT116 colon cancer cell line, were cultured *in vitro* and treated with increasing concentrations of 5 polyphenols for 48 h, respectively, namely resveratrol (Res), piceatannol (Pic), pterostilbene (PTS), trimethylstilbene (TriMRes) and myricetin. Following treatment, the cells were harvested and stained for the surface expression of PD-L1 by flow cytometry. The geometric mean of mean fluorescent intensity (MFI) of phytoerythrin (PE) area was used as the readout of PD-L1. The levels of PD-L1 were converted to a bar graph to represent the respective changes in PD-L1 expression following treatment. The parental condition (also referred to as DMSO-treated, or control cells). Statistical difference reflects the comparison of treated samples to the parental condition. The data shown were from n=3 independent experiments. *P<0.05.

To determine whether the upregulation of PD-L1 by resveratrol and piceatannol was broadly or uniquely observed in specific breast or colon cancer cell lines, we assayed any alterations in PD-L1 expression using a panel of breast (Cal51, BT549, BT474 and SKBR3) and colorectal (HCT116, SW480,

A



B

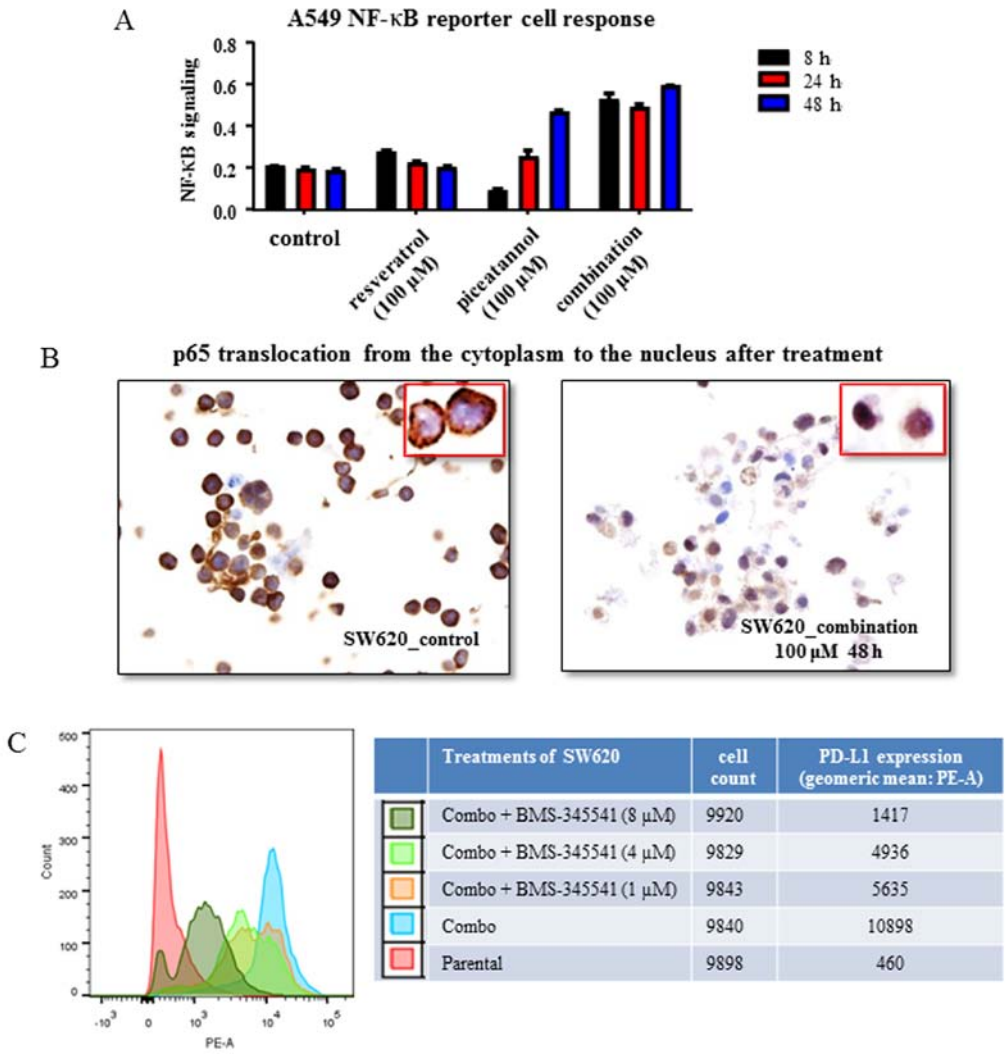
Cell line	Indication	mRNA (TPM)
		PD-L1
Cal51	Breast	0.138
BT474	Breast	1.024
SKBR3	Breast	1.679
BT549	Breast	3.286
SW620	Colon	0.234
HCT116	Colon	0.925
HT29	Colon	1.543
SW480	Colon	2.146

** P=0.001
* P=0.05
x P=0.05 (treated vs. control)

Figure 2. Effects of resveratrol and piceatannol on programmed cell death ligand 1 (PD-L1) expression in human breast and colon cancer cells. (A) A panel of 4 breast (Cal51, BT549, BT474 and SKBR3) and colorectal (HCT116, SW480, HT29 and SW620) cancer cell lines were incubated with increasing concentrations of resveratrol or piceatannol, as single agents or in combination for 48 h. Following treatment, the cells were harvested and stained and quantified for PD-L1-PE surface expression by flow cytometry. The induction of PD-L1 expression was based on comparison to a DMSO-treated sample (control used throughout all experiments), and to isotype control staining. The geometric mean of MFI of PE area was used as the PD-L1 readout. The levels of PD-L1 from n=3 experiments were converted to a bar graph to represent the respective changes in PD-L1 expression following treatment. The parental condition represents the untreated control. The statistical difference reflects comparison of treated samples to parental condition. (B) RNA-seq analysis of constitutive mRNA expression of PD-L1 in panel of breast and colon cancer cells. Basal PD-L1 mRNA levels, shown as transcript per million (TPM) were obtained from a public Cancer Cell Line Encyclopedia (CCLE) database and ranked from low to high according to the relative TPM expression by most of the breast and colon cancer cell lines tested in (A) https://portals.broadinstitute.org/ccle/home. The asterisks apply to comparisons made among treated samples: *P<0.05, **P<0.01, ***P<0.001. In addition, the 'x' symbol refers to comparisons made between the untreated, control sample with the treated sample: xP<0.05 (treated vs. control).

HT29 and SW620) cancer cell lines. In addition, we also determined whether the synergistic upregulation of PD-L1 may result from treatment with the two stilbenoids. The differential increase in PD-L1 expression induced by resveratrol or piceatannol was observed in 2/4 breast and 3/4 colorectal cancer cell lines treated with either of the stilbenoids as a single agent (Fig. 2A). The combination of resveratrol and piceatannol acted synergistically; 50 μM each of resveratrol and piceatannol combined and referred to as 'combo-100' resulted in significantly greater induction of PD-L1 expression; specifically, ≥4.5-fold in the Cal51 and ≥3.5-fold in the SW620 cells than 50 μM of either stilbenoid added alone (Fig. 2A). Gene expression analyses frequently reveal that the relative abundance of mRNA is only weakly or even inversely associated with the level of protein expression (49-51). Thus, in this study, to determine whether the differential expression level of endogenous PD-L1 mRNA might contribute to the observed induction of PD-L1 by resveratrol and piceatannol in these two cell lines, relative to the panel of the other studied cell lines with the same cancer type grouping, the basal PD-L1 mRNA expression levels, shown as TPM were obtained from a public Cancer Cell Line Encyclopedia (CCLE, https://portals.broadinstitute.org/ccle/home) database for cancer cell lines tested. In the breast cancer cell lines, the endogenous level of PD-L1 mRNA ranked as follows: Cal51 ≤ BT474 < SKBR3 ≤ BT549 (Fig. 2B); however, the induction of PD-L1 by co-treatment with resveratrol and piceatannol yielded the opposite result: Cal51 (~7-fold) ≥ BT474 (~ 4-fold) > SKBR3 (~2-fold) ≥ BT549 (~2-fold) (Fig. 2A). A similar trend was also observed in the colorectal cancer cells; namely, the endogenous PD-L1 mRNA ranking was as follows: SW620 ≤ HCT116 < HT29 ≤ SW480 (Fig. 2B), whereas the relative induction of PD-L1 decreased from high to low as follows: SW620 (~4-fold) > HCT116 (~3-fold) > SW480 (~3-fold) > HT29 (~1.5-fold) (Fig. 2A). These results suggest that tumors with a low endogenous mRNA level of PD-L1 are more likely to be affected by resveratrol and/or piceatannol, alone or in combination.

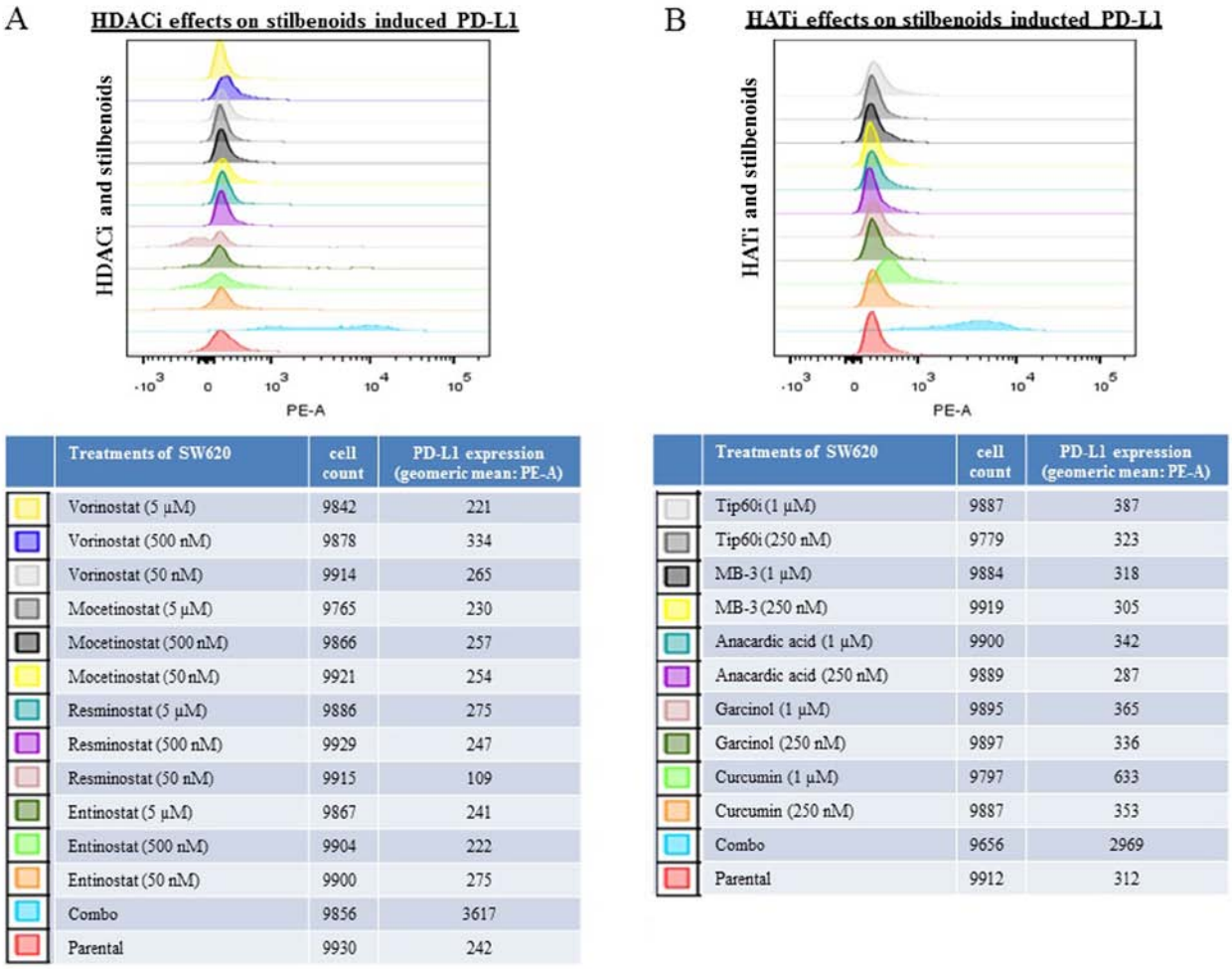
NF-κB mediates the upregulation of PD-L1 induced by resveratrol and/or piceatannol. IFN-γ is known to induce



	Treatments of SW620	cell count	PD-L1 expression (geomeric mean: PE-A)
	Combo + BMS-345541 (8 μM)	9920	1417
	Combo + BMS-345541 (4 μM)	9829	4936
	Combo + BMS-345541 (1 μM)	9843	5635
	Combo	9840	10898
	Parental	9898	460

Figure 3. Control of nuclear factor (NF)-κB-mediated programmed cell death ligand 1 (PD-L1) expression by resveratrol and piceatannol. (A) A549 dual reporter cells were used to assay the activation of NF-κB following treatment with resveratrol or piceatannol, each at 100 μM or combined, each at 50 μM (referred to as combo-100) to yield a concentration of 100 μM. Resveratrol as a single agent resulted in an optimal early time activation of NF-κB, while piceatannol alone decreased NF-κB signaling at 8 h and followed by a progressive increases at 24 and 48 h, respectively. When used in combination, the two stilbenoids led to a marked increase in NF-κB signaling over the tested duration of 48 h. The parental condition represents the untreated control. The data shown are from 1 experiment. (B) Immunohistochemistry of the subcellular localization of NF-κB p65 subunit in SW620 colon cancer cells treated with a combination of piceatannol and resveratrol, each at 50 μM to yield a concentration of 100 μM vs. the untreated cells. The analysis showed that p65 translocated from the cytoplasm to the nucleus by the combined use of the stilbenoids, indicating the activation of NF-κB signaling. Images were captured at x20 magnification and cropped to show the field of cells representative of the effect of treatment. The results are representative of 5 sections from 1 experiment. We did not quantify the percentage of cells in which translocation occurred in these samples. The parental condition represents the control. The image in the inset represents a high magnification image of the cells in the field of view. (C) BMS-345541, an inhibitor of IKK, was administered for 24 h *in vitro* prior to exposure to the combination of piceatannol and resveratrol, each at 50 μM to yield a concentration of 100 μM, for a further 48 h. BMS-345541 was added at increasing concentrations (1, 4 and 8 μM) while the dose of the combination was kept constant. Following treatment, the cells were harvested and stained for PD-L1 expression by flow cytometry. The geometric mean of the mean fluorescent intensity (MFI) of the phytoerythrin (PE) area was used as the readout for PD-L1 expression. The parental condition represents the untreated control. The results are representative of 3 experiments.

PD-L1 expression by upregulating its transcription through the activation of interferon regulatory transcription factor (IRF-1) (52,53). Thus, in this study, we investigated whether the same mechanism may contribute to the resveratrol- and/or piceatannol-mediated induction of PD-L1 expression. The results of immunohistochemistry revealed no change in IRF-1 expression in the SW620 cells treated for 48 h with 'combo-100' compared to the control (data not shown). Since the NF-κB consensus sequence is also present in the PD-L1 gene promoter (52,53), and NF-κB plays a major role in the transcription of PD-L1 by IFN-γ (54-56), in this study, we examined whether the induction of PD-L1 by resveratrol and/or piceatannol was due to the activation of NF-κB. The A549 cells co-expressing the SEAP reporter gene and Lucia luciferase gene were used to investigate the association between NF-κB activity and the PD-L1 expression levels following treatment with the two stilbenoids, alone or combination. In this dual reporter assay, the secreted SEAP and Lucia luciferase in the culture supernatant were separately measured to provide a quantitative readout of the transcriptional impact of NF-κB and the IFN signaling pathways. The time-dependent (≥24 h) and synergistic induction of NF-κB expression induced by piceatannol alone and by combined treatment with resveratrol was observed (Fig. 3A). In response to stimuli, the



	Treatments of SW620	cell count	PD-L1 expression (geomeric mean: PE-A)
	Vorinostat (5 µM)	9842	221
	Vorinostat (500 nM)	9878	334
	Vorinostat (50 nM)	9914	265
	Mocetinostat (5 µM)	9765	230
	Mocetinostat (500 nM)	9866	257
	Mocetinostat (50 nM)	9921	254
	Resminostat (5 µM)	9886	275
	Resminostat (500 nM)	9929	247
	Resminostat (50 nM)	9915	109
	Entinostat (5 µM)	9867	241
	Entinostat (500 nM)	9904	222
	Entinostat (50 nM)	9900	275
	Combo	9856	3617
	Parental	9930	242

	Treatments of SW620	cell count	PD-L1 expression (geomeric mean: PE-A)
	Tip60i (1 µM)	9887	387
	Tip60i (250 nM)	9779	323
	MB-3 (1 µM)	9884	318
	MB-3 (250 nM)	9919	305
	Anacardic acid (1 µM)	9900	342
	Anacardic acid (250 nM)	9889	287
	Garcinol (1 µM)	9895	365
	Garcinol (250 nM)	9897	336
	Curcumin (1 µM)	9797	633
	Curcumin (250 nM)	9887	353
	Combo	9656	2969
	Parental	9912	312

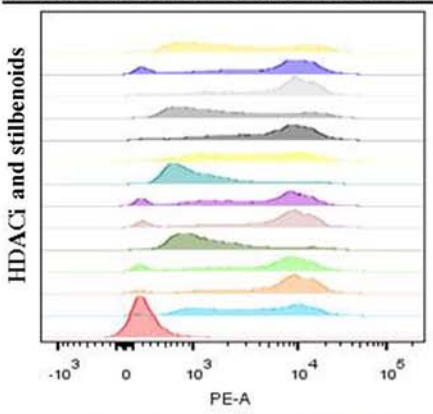
Figure 4. Effects of histone deacetylase inhibitors (HDACis) and histone acetyltransferase inhibitors (HATis) on the induction of programmed cell death ligand 1 (PD-L1) expression. (A) The SW620 colon cancer cell line was treated *in vitro* with different classes of HDACis at various concentrations for 72 h. Following treatment, the cells were harvested and stained for PD-L1 expression by flow cytometry. The results were quantified using the geometric mean of the mean fluorescent intensity (MFI) of the phytoerythrin (PE) area as the readout for the expression of PD-L1. (B) The same cancer cell line, SW620, was treated with a known class of HATis listed, for 72 h and PD-L1 expression was analyzed and quantified. 'Combo' indicates treatment with both resveratrol and piceatannol each at 60 μM for 48 h. The parental condition represents the untreated control. The data shown are from 1 experiment.

inhibitory protein IκB is degraded, which leads to the release/translocation of heterodimer p65/p50 from the cytoplasm to the nucleus (57,58). Therefore, the activation of NF-κB by the combined stilbenoids was examined in the SW620 cells by immunohistochemistry to analyze changes in the localization of p65; 48 h of exposure to 'combo-100' resulted in an increase in the translocation and nuclear accumulation of p65, as shown in Fig. 3B (inset).

The small molecule, BMS-345541, is an IKK kinase inhibitor (59) that prevents IκB phosphorylation, to effectively suppress the translocation of NF-κB into the nucleus for participation in transcriptional activation of NF-κB-responsive genes (57,58). Thus, in this study, we then examined whether the stilbenoid-induced PD-L1 expression can be blocked by BMS-345541. In SW620 cells, the induction of PD-L1 by 'combo-100' was inhibited by 48% with 1 μM BMS-345541 (Fig. 3C). Furthermore, a dose-dependent inhibition was observed with the 1, 4 and 8 μM of IKK inhibitor concentration range (Fig. 3C). These results, showing that the inhibition of IKK significantly decreased PD-L1 expression in SW620 cells suggest that NF-κB activation is involved in the induction of PD-L1 expression by resveratrol or piceatannol either alone, or in combination.

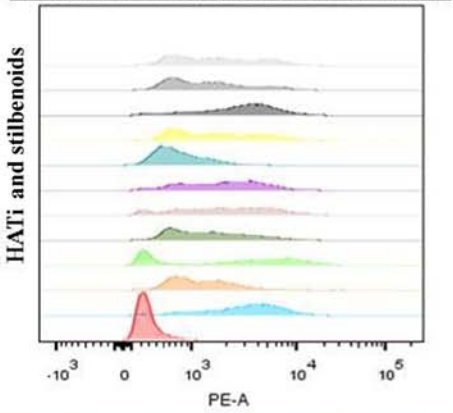
Histone deacetylase inhibitors (HDACis) and histone acetyltransferase inhibitors (HATis) modulate the induction of PD-L1 expression induced by the combination of resveratrol and piceatannol. The expression of PD-L1 can be regulated via histone acetylation/deacetylation (60,61) and resveratrol is an activator of HDAC (62). To investigate whether the induction of PD-L1 by the combination of resveratrol and piceatannol is blocked by inhibitors of HDAC or HAT, HDACis (vorinostat, mocetinostat, resminostat and entinostat) and HATis (curcumin, garcinol, anacardic acid, MB-3 and Tip60i) were used to assess their effects on the modulation of PD-L1 by the combined use of the stilbenoids. The cells were pre-treated for 48 h with individual HDACis/HATis alone or in combination with 60 μM of either of the stilbenoids, followed by the flow cytometric analysis of PD-L1 expression. The addition of HDACis or HATis alone did not affect PD-L1 expression compared to the untreated controls (Fig. 4). When the SW620 cells were pre-treated with histone modification inhibitors,

A



Treatments of SW620	cell count	PD-L1 expression (geomeric mean: PE-A)
Combo + Vorinostat (5 μM)	9830	2651
Combo + Vorinostat (500 nM)	9789	4144
Combo + Vorinostat (50 nM)	9810	5614
Combo + Mocetinostat (5 μM)	9757	2321
Combo + Mocetinostat (500 nM)	9711	5004
Combo + Mocetinostat (50 nM)	9813	3353
Combo + Resminostat (5 μM)	9838	1377
Combo + Resminostat (500 nM)	9806	3454
Combo + Resminostat (50 nM)	9814	4560
Combo + Entinostat (5 μM)	9788	1679
Combo + Entinostat (500 nM)	9791	4032
Combo + Entinostat (50 nM)	9860	5507
Combo	9856	3617
Parental	9930	242

B

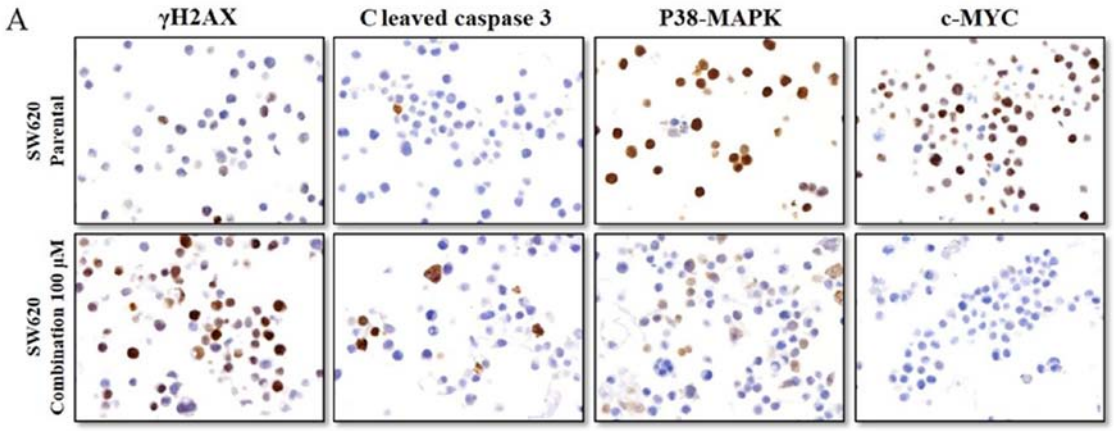


Treatments of SW620	cell count	PD-L1 expression (geomeric mean: PE-A)
Combo + Tip60i (1 μM)	9602	1806
Combo + Tip60i (250 nM)	9700	1510
Combo + MB-3 (1 μM)	9430	2698
Combo + MB-3 (250 nM)	9656	1841
Combo + Anacardic acid (1 μM)	9802	850
Combo + Anacardic acid (250 nM)	9595	2013
Combo + Garcinol (1 μM)	9613	2106
Combo + Garcinol (250 nM)	9687	1594
Combo + Curcumin (1 μM)	9379	2209
Combo + Curcumin (250 nM)	9381	1404
Combo	9656	2969
Parental	9912	312

Figure 5. Effects of histone deacetylase inhibitors (HDACis) and histone acetyltransferase inhibitors (HATis) on the induction of programmed cell death ligand 1 (PD-L1) expression by a combination of resveratrol and piceatannol. (A) The SW620 colon cancer cell line was treated *in vitro* with increasing concentrations of HDACis for 24 h prior to exposure to a combination of resveratrol and piceatannol, each at 60 μM, for an additional 48 h. Following treatment, the cells were harvested and stained for PD-L1 expression by flow cytometry. The geometric mean of the mean fluorescent intensity (MFI) of the phytoerythrin (PE) area was used as the readout for PD-L1 expression. The high dose of entinostat and resminostat significantly reduced expression of PD-L1. (B) The SW620 cells were treated with listed HATis, for 24 h prior to exposure to the combined treatment as described in Fig. 3A. The analysis and quantification of PD-L1 were identical to those shown in Fig. 3A. 'Combo' indicates treatment with both resveratrol and piceatannol each at 60 μM for 48 h. The parental condition represents the untreated control.

the ability of the stilbenoids to induce PD-L1 was markedly reduced by two HDACis (5 μM of resminostat and entinostat) and also by the HATi, anacardic acid (1 μM) (Fig. 5). These results demonstrated that histone modification inhibitors can suppress the induction of PD-L1 expression by stilbenoids. The data are consistent with the interpretation that the upregulation of PD-L1 by stilbenoids involves transcriptional control.

Induction of apoptotic and cell cycle changes by the combined use of resveratrol and piceatannol. The upregulation of PD-L1 may allow cancers to evade the host immune system and acquire resistance to anticancer drugs. Having demonstrated that the upregulation of PD-L1 expression by stilbenoids in the SW620 colon cancer cells, we then investigated whether stilbenoids affect the survival status of cells by analyzing two biomarkers related to apoptosis, namely, the expression of the DNA damage indicator γH2AX, and that of cleaved caspase 3. In addition, markers associated with cell survival, p38-MAPK and c-Myc, were also assessed using immunohistochemistry. Treatment of the SW620 cells for 48 h with 'combo-100' resveratrol and piceatannol increased the expression of γH2AX and that of cleaved caspase 3, and downregulated the p38-MAPK and c-Myc levels (Fig. 6A). The induction of γH2AX is characteristic of DNA fragmentation and damage during apoptosis, and thus supports the interpretation that exposure to resveratrol and/or piceatannol causes DNA damage and apoptosis via the activation of caspase 3. We then assessed whether treatment with the stilbenoids altered cell cycle distribution by flow cytometric analysis. An increase in the percentage of cells in the S phase of the cell cycle, from 19 to ~30%, a distinct reduction in the proportion of G_1 phase cells (from 66 to ~30%), and a marked decrease in the percentage of G_2M phase cells, from 12 to ~3% were observed. There was also an increase in the percentage of cells with fractional DNA content ('sub-G_1 cells'), an indication of apoptosis from 2.85% in the control cells to 31.84, 35.08 and 36.55% in the cells treated with



B

Treatment	Sub-G1	G1	S	G2/M
Control (C)	2.85	65.64	19.17	12.34
50 µM resveratrol (R)	31.84	30.45	34.98	2.73
50 µM piceatannol (P)	35.08	33.86	29.59	1.47
50 µM combination (Combo)	36.55	26.61	33.89	2.95

Figure 6. Effects of resveratrol and/or piceatannol on the cell survival and cell cycle status. (A) Immunohistochemical staining of SW620 parental cells, top row, and cells treated with the combination of resveratrol and piceatannol, each at a concentration of 50 μM, are shown on the bottom row. Cells from both conditions were stained with antibodies against γH2AX, cleaved caspase 3, p38 MAPK and c-Myc, as described in the Materials and methods. Images were captured at x20 magnification and cropped to show the field of cells representative of the effect of treatment. (B) Flow cytometric analysis of the changes on cell cycle status and apoptotic index of SW620 cells. The percentage of cells in particular phases of the cell cycle identified by their DNA content is indicated. The data are shown from a single representative experiment.

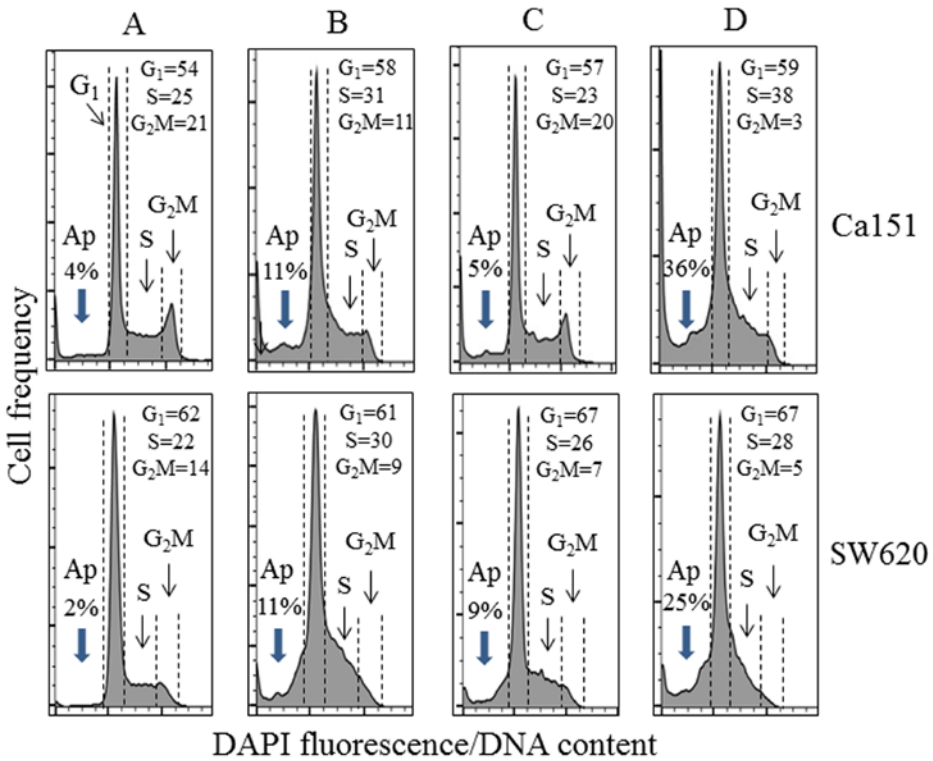


Figure 7. Flow cytometric analysis of the changes in cell cycle status and the apoptotic index of Cal51 and SW620 cells. (A) Untreated cells. (B) Cells treated with 10 μM resveratrol, 48 h. (C) Cells treated with 10 μM piceatannol, 48 h. (D) Cells treated with 10 μM of resveratrol plus 10 μM piceatannol, 48 h. The percentage of apoptotic cells (Ap) identified by fractional DNA content, sub-G_1 cells is shown in each panel (thick arrows). The percentage of cells in particular phases of the cell cycle identified by their DNA content, are as shown by the thin arrows in the top panel (Ca151 cells) and the bottom panel (SW620 cells). The data shown are representative of two experiments.

50 μM resveratrol, 50 μM piceatannol or 50 μM of these two compounds for 48 h, respectively (Fig. 6B). We also examined and determined that low-dose (10 μM) resveratrol and/or piceatannol did not induce PD-L1 expression, whereas the analysis of cell cycle phase distribution changes using Cal51 and SW620 cell cultures treated for 48 h revealed an apparent increase in the proportion of S-phase cells concomitant with the reduction of G_2M-phase cells in cultures treated with resveratrol alone, and to an even greater extent following treatment with both stilbenoids (Fig. 7). In addition, the combination of resveratrol and piceatannol induced apoptosis in both cell types to a much greater degree than each of them alone, as demonstrated by an increase in the percentage of cells with fractional DNA content ('sub-G_1 cells'), an indication of apoptosis (compare Fig. 7A with Fig. 7B-D) (48,63,64). Taken together, our findings indicate that stilbenoids not only increase PD-L1 expression, but may also induce DNA damage, leading to increased cell death in tumor cells, such as SW620 colon cancer cells.

Discussion

An association has been observed between a decrease in T-cell proliferation and an increase in apoptosis and tumor immune evasion, with an increase in the expression of T-cell inhibitory protein PD-L1 on cancer cells (2,3), providing the impetus for understanding the control of PD-L1 expression using cancer cells as a model. In this study, we examined the hypothesis that dietary stilbenoids may act as modulators of PD-L1 expression in tumor cells. We focused on the effects of resveratrol, a grape-derived polyphenol that has shown chemopreventive effects in various cancer types (33-42), and its hydroxylated derivative, piceatannol. Questions raised and addressed in this study included whether: i) Structurally-modified stilbenoids have the same ability to modulate PD-L1 expression; ii) the effects of stilbenoids on PD-L1 expression can apply to different cancer types; iii) signaling pathways that control the stilbenoid-induced PD-L1 expression are effected; and iv) histone modification inhibitors (HDACis and HATis) block stilbenoid-induced PD-L1 expression.

Stilbenoids in general exert beneficial effects on human health (65). It has been reported that the 4'-hydroxy group of resveratrol is essential for its bioactivity (66). In this study, we used breast and colorectal cancer cells to compare the modulatory effects of stilbenoids on PD-L1 expression, namely, piceatannol (3',4',3,5-trans-trihydroxystilbene), 3,5,4'-trimethoxystilbene, and pterostilbene (3,5-dimethoxy-4'-hydroxy-E-stilbene), with resveratrol (3,5,4'-trans-trihydroxystilbene) and myricetin, a naturally occurring flavonoid present in abundance in edible foods (67). The results presented in Figs. 1 and 2 demonstrate that the hydroxyl, but not methoxy groups on stilbenoids are important for the induction of PD-L1. However, no increase in PD-L1 expression was evident in the cells treated with myricetin (3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-4-chromenone), a flavonoid that contains 6 hydroxyl groups (Fig. 1). These results suggest that the 3,5-dihydroxy-trans-stilbene structure is required for the induction of PD-L1 in the cells used.

The expression level PD-L1 of was significantly increased in the breast and colon cancer cells treated with resveratrol and piceatannol, both as a single agent and when used in combination (Figs. 1 and 2). Moreover, PD-L1 expression was synergistically upregulated 4.5-fold in the Cal51 breast cancer and ≥3.5-fold in the SW620 colon cancer cells by the combined use of resveratrol and piceatannol (Fig. 2A). Of note, both cancer cells express low basal levels of PD-L1 mRNA expression (Fig. 2B). Since, as noted, a positive response and improved clinical outcome to anti-PD-L1 blockade therapy are best observed in patients with PD-L1-positive tumors (presumably reflecting a high expression of PD-L1), we surmise that agents capable of upregulating PD-L1 expression in tumor cells expressing low PD-L1 can sensitize such cancer cells for an improved response to PD-L1 blockade. As a corollary, we also hypothesized that the combined use of resveratrol and piceatannol co-administered with anti-PD-L1 immunotherapy may exhibit clinical benefits in cancer patients with no-or-low-PD-L1 tumors. Whether the efficacy to PD-L1 blockade may be enhanced by the combined intake of resveratrol and/or piceatannol, concomitantly or sequentially, remains to be verified.

This study also provides evidence showing that the resveratrol- and piceatannol-mediated upregulation of PD-L1 requires the activation of NF-κB (Fig. 3). In the SW620 cells, we showed that the induction of PD-L1 expression induced by resveratrol/piceatannol was attenuated by the IKK inhibitor, BMS 345541 (Fig. 3C). It has been previously demonstrated that the duration and function of nuclear NF-κB is regulated by reversible acetylation/deacetylation (68), and that NF-κB transcriptional response is controlled by the HDAC3-mediated deacetylation of RelA acting as an intranuclear molecular switch for turning 'on-off' the NF-κB response (68); we therefore examined whether histone modification inhibitors affect the resveratrol/piceatannol-mediated transcriptional control of PD-L1. Our findings revealed that two HDACis (e.g., resminostat and entinostat) and one HATi, anacardic acid, effectively blocked the induction of PD-L1 expression by resveratrol/piceatannol (Fig. 5). Both resminostat and entinostat are HDAC3 inhibitors, thus lending support that the HDAC3-mediated NF-κB response plays a role in resveratrol/piceatannol-induced PD-L1 expression. The suppression of PD-L1 induction by resveratrol using anacardic acid is in accordance with the described effect of anacardic acid as a HATi for p300 and p300/CBP and data reporting that resveratrol is a p300 activator (69,70). These results indicate that the expression of PD-L1 is regulated by the mechanism of histone acetylation/deacetylation and that resveratrol/piceatannol induces PD-L1 expression through HDAC3/p300-mediated NF-κB control.

It should be mentioned that the upregulation of PD-L1 by resveratrol or piceatannol occur at doses not achievable physiologically and may exceed pharmacologically relevant concentrations (26,71). Conceivably, the effective dose could also be modulated by factors present locally at the site of responsive cells/tumors (e.g., different hormones, cytokines, products of cell metabolism or variable oxygen tension) and thus may additionally affect sensitivity of PD-1/PD-L1 checkpoint to these compounds, perhaps amplifying their potential anticancer effect. It should also be noted that the doses used in the present experiments were based on titration studies (data not shown), and that the effectiveness of single or combined agents on the induction of PD-L1 in each cell line is above IC_{50}. It would be of interest to determine what might

account for the variations observed in dosage dependence in different cell lines. Since the induction of PD-L1 expression by resveratrol and piceatannol are mediated through the NF-κB signaling pathway; the different dose-dependent responses and the upregulation of PD-L1 by resveratrol and piceatannol may be due to the variation in the endogenous level of NF-κB components, vis-à-vis, NF-κB1 (p105), NF-κB2 (p65), CHUK (IKK-α), IKBKB (IKK-β) and IKBKG (IKK-γ), in each of the cell lines tested. In RNA-seq analyses, we found that Cal51 and SW620, both with a low endogenous level of PD-L1 expression, expressed high levels NF-κB2 (p65) and CHUK (IKK-α) compared to cancer cell lines from same anatomical origin showing high PD-L1 expression (data not shown). Thus, it is tempting to propose that response in the induction of PD-L1 by stilbenoids in different cell lines from identical cancer types may be attributed to the level of expression of NF-κB2 (p65) and CHUK (IKK-α). Currently, experiments are underway to further test and confirm our hypothesis.

Another result of note in this study is that the SW620 cells exposed to a high dose of both stilbenoids were partially restricted in cell cycle transition in the G_2/M phase and display evidence of apoptosis (Fig. 6). Furthermore, the accumulation of cells in the S phase of the cell cycle may also be associated with an increase in their expression of PD-L1. This suggests that resveratrol and piceatannol affect cancer cells by a dual mechanism: i) The induction of PD-L1 that sensitizes tumor cells for recognition by anti-PD-L1 antibodies; an effect that could diminish cancer cell evasion from immune surveillance; and ii) the direct induction of cell cycle arrest, increase in DNA damage and cancer cell destruction via induction of apoptosis.

Since cancer patients expressing tumors positive for PD-L1, a negative T-cell regulatory molecule, demonstrate efficacy to anti-PD-L1 blockade therapy with an improved clinical outcome, one might surmise that low PD-L1-expressing tumors may be sensitized and may display an improved responsiveness to PD-L1 blockade therapy using dietary agents. The cell culture experiments used in this study may be considered as a model for testing whether the sensitivity and responsiveness of tumor cells to PD-L1 targeted therapy can be augmented by priming with or co-exposure to stilbenoids, such as resveratrol and/or piceatannol. The hypothesis raised is as follows: The upregulation of membrane-associated PD-L1 in low PD-L1-expressing tumor cells is a 'find-me' approach for targeting by immune checkpoint inhibitors to potentially improve the efficacy of anti-PD-L1 blockade therapy via stilbenoids. Indeed, we believe that the elevation of PD-L1 expression, as we have demonstrated in this study using pharmacological doses of the stilbenoids, resveratrol and piceatannol, may underlie the unresolved challenge in that the positive response to immune checkpoint blockade therapy in 19-31% of treated individuals is a limited to number of clinical indications, typically in patients whose tumors express elevated-PD-L1, which we stated explicitly in the Introduction. Thus, while on teleological grounds, the upregulation of PD-L1 by polyphenols in cancer could promote disease progression, we offer the consideration that the observed stilbenoid-induced PD-L1 increase be viewed from the hypothesis that agents capable of upregulating PD-L1 expression in tumor cells could sensitize cancer cells for an improved clinical response to PD-L1 immune checkpoint blockade therapy. Testing these aspects would constitute a novel approach to confirm our hypothesis. These possibilities are under further investigative considerations in our laboratory. Studies are also planned to explore whether stilbenoids may impact host immune response, for example, by affecting PD-1 expression in PD-1-expressing Jurkat T-cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JL performed experiments and analyzed the data (cell culture analysis, immunohistochemistry, flow cytometric analysis, NF-κB reporter assay and statistical analysis) and edited the manuscript. TCH interpreted the data and wrote the manuscript. HDH collected and analyzed/interpreted the data (cell cycle and apoptosis analyses). ZD interpreted the data and wrote the section of cell cycle and apoptosis analyses and edited the manuscript. JMW interpreted the data, and wrote and edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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