The prednisolone phosphate-induced suppression of the angiogenic function of tumor-associated macrophages enhances the antitumor effects of doxorubicin on B16.F10 murine melanoma cells *in vitro*

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Abstract. Several lines of evidence have clearly demonstrated the role of the tumor microenvironment in favoring the drug resistance of melanoma cells, as well as the progression of this cancer type. Since our previous studies proved that the accumulation of prednisolone disodium phosphate (PLP) in melanoma tissue inhibited tumor growth by exerting anti-angiogenic effects on the most abundant cells of the tumor microenvironment, tumor-associated macrophages (TAMs), the present study investigated whether PLP could enhance the cytotoxic effects of doxorubicin (DOX) on B16.F10 murine melanoma cells. To assess the antitumor efficacy of the combined therapeutic approach based on PLP and DOX, we used a co-culture system composed of bone marrow-derived macrophages (BMDMs) and B16.F10 murine melanoma cells at a cell density ratio that approximates the melanoma microenvironment *in vivo*, ensuring the polarization of the BMDMs into TAMs. Thus, we assessed the combined therapeutic effects of PLP and DOX on melanoma cell proliferation and apoptosis, as well as on supportive processes for tumor growth, such as oxidative stress as well as the angiogenic and inflammatory capacity of the cell co-culture. Our data demonstrated that the cytotoxicity of DOX was potentiated mainly via the anti-angiogenic activity of PLP in the melanoma microenvironment *in vitro*. Moreover, the amplitude of the cytotoxicity of the combined treatments may be linked to the degree of the suppression of the pro-angiogenic function of TAMs. Thus, the potent decrease in the expression of the majority of the angiogenic and inflammatory proteins in TAMs following the concomitant administration of PLP and DOX may be associated with their anti-proliferative, as well as pro-apoptotic effects on B16.F10 melanoma cells. However, the combination therapy tested did not affect the immunosuppressive phenotype of the TAMs, as the levels of two important markers of the M2-like phenotype of macrophages (IL-10 and Arg-1) were not reduced or even increased following these treatments. On the whole, the findings of this study indicated that PLP improved the therapeutic outcome of DOX in the melanoma microenvironment via the inhibition of the pro-angiogenic function of TAMs.

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Introduction

Metastatic melanoma is one of the most challenging types of cancer to treat due to its aggressiveness and resistance to treatment. Current progress in the understanding of the molecular mechanisms responsible for tumor growth and metastasis has led to the approval of several novel therapies for melanoma; however, the adverse effects associated with the treatments and the development of drug resistance in a relative short period of time have underscored their benefit (1-9). Recent studies have clearly demonstrated that the inflammatory tumor microenvironment favors innate and acquired resistance of cancers cells to cytotoxic drugs altering the treatment outcome (10-13). Thus, therapeutic approaches based on the anti-neoplastic polarization of the tumor microenvironment may be promising strategies with which to overcome drug resistance in melanoma, as well as in general in solid tumors (14,15).

Among the innate immune cells present in the stroma of solid tumors, macrophages [tumor-associated macrophages (TAMs)] are the most abundant, representing up to 50% of the tumor mass (16). Thus, TAMs are polarized into the M2 phenotype and are most important in promoting and supporting tumor inflammation, as well as in all processes dependent on chronic inflammation, such as angiogenesis,

tumor growth and metastasis, and immunosuppression (17-19). Several studies have clearly demonstrated the role of TAMs in promoting tumor angiogenesis via the secretion of several pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β). Moreover, a high number of TAMs infiltrated into human tumors is associated with an increased microvessel density in different types of human cancer (20-24). In tight association with these findings, we have previously demonstrated that TAMs are the most important tumor stromal cells that support melanoma tumor growth *in vivo* through the production of pro-angiogenic proteins (25,26). Consequently, the depletion of TAMs from melanoma tumors markedly inhibits tumor growth (50% of tumor growth inhibition compared to the growth of controls) via the suppression of the pro-angiogenic capacity of these cell types (25,26). Therefore, anti-inflammatory drugs that disrupt the link between TAM-induced inflammation/angiogenesis and tumor growth may provide new therapeutic opportunities against cancer. Thus, the well-known anti-inflammatory drug, prednisolone disodium phosphate (PLP), administered in liposomal form, has been shown to exert potent antitumor effects on B16.F10 melanoma-bearing mice via the inhibition of the angiogenic/inflammatory capacity of TAMs (26-28). Additionally, it has been demonstrated that TAMs can protect tumor cells against the cytotoxicity induced by chemotherapeutic drugs, such as doxorubicin (DOX) and etoposide (29). DOX is a potent cytotoxic drug used in the treatment of various types of cancer (30). Although DOX exerts a potent cytotoxic effect on several melanoma cell lines, its use as a first-, as well as second-line therapy for melanoma, is well-tolerated, although it is limited by a low clinical efficacy, suggesting a modulatory activity of the tumor microenvironment (31-33).

Based on these findings, the present study aimed to examine whether prednisolone that targets the antitumor functions of TAMs, can enhance the cytotoxicity of the DOX on B16.F10 melanoma cells, when both agents are co-administered. To assess the antitumor efficacy of the combined treatment, we used an *in vitro* model for the inflammatory melanoma microenvironment based on the co-culture of bone marrow-derived macrophages (BMDMs) and B16.F10 murine melanoma cells at a cell density ratio of 4:1. This ratio approximates the murine melanoma microenvironment *in vivo* that ensures the polarization of BMDMs into TAMs (34). Thus, we examined the effects of the combined treatment on main processes that can affect melanoma development and aggressiveness. In this respect, we assessed the combined therapeutic effects on cancer cell proliferation and apoptosis, as well as on supportive processes for tumor growth, such as oxidative stress, as well as on the angiogenic and inflammatory capacity of the co-culture. To gain insight into the mechanisms through which this treatment can influence the protumor functions of TAMs, the effects on key molecules produced by this cell type and which are involved in the main processes that support tumor development, such as tumor inflammation and angiogenesis, were also screened. The results revealed that co-treatment with DOX and PLP exerted enhanced antitumor effects compared to treatment with the cytotoxic drug alone, mainly due to the PLP-induced inhibition of the angiogenic activity of TAMs.

Materials and methods

Cell types and culture conditions. B16.F10 murine melanoma cells (CRL-6475; American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza Group Ltd.), supplemented with 10% heat-inactivated fetal bovine serum (HyClone; GE Healthcare Life Sciences), 100 IU/ml penicillin, 100 μg/ml streptomycin and 4 mM L-glutamine (Lonza Group Ltd.) as monolayer at 37˚C in a 5% CO_2 humidified atmosphere.

Bone marrow cells were isolated from the femurs of male C57BL/6 mice (Cantacuzino Institute, Bucharest, Romania) following a previously published protocol (35). The adherent BMDMs were harvested after 7 days of cultivation in medium supplemented with 10 ng/ml macrophage colony-stimulating factor (M-CSF; Cell Signaling Technology). For the co-culture model, the BMDMs were cultured with B16.F10 cells at a cell density ratio of 4:1 for an optimal cytokine exchange and interaction specific for *in vivo* melanoma microenvironment (34,36). When used in monoculture, the differentiated macrophages were polarized toward the M2 phenotype by supplementation of the growth medium with 20 ng/ml interleukin (IL)-4 (Cell Signaling Technology) for 24 h. Experiments were performed according to the European and national regulations and were approved by the Committee on the Ethics of Animal Experiments of the Babes-Bolyai University (registration no. 31444/27.03.2017).

Stock solutions of DOX and PLP. DOX (Sigma-Aldrich, cat. no. D2975000) and PLP) (Sigma-Aldrich, cat. no. 1557000) were dissolved in sterile water to prepare stock solutions of 10 and 100 mM, respectively. Working solutions were prepared directly into the culture media.

Cell proliferation assay. To determine the effects of DOX, administered alone or in combination with PLP, on tumor cell proliferation, the B16.F10 melanoma cells (1,000 cells/well) co-cultured with BMDMs (4,000 cells/well) were seeded in a 96-well plate for 24 h. Various concentrations of DOX (ranging from 0.007-0.5 μM) and PLP (ranging from 2.5-20,000 μM) were administered alone and tested in triplicate to assess the IC_{50} values. Based on our previous studies (28,37), the concentration of 410 μM of PLP was selected to be administered in combination with various concentrations of DOX on the cell co-culture. Although this concentration of PLP does not exert any significant anti-proliferative effects on melanoma cells, it has been proven to exhibit antitumor activity mediated by its inhibitory effects on the angiogenic and inflammatory proteins produced by TAMs (28,37). Cell co-culture incubated only with medium was used as a control. The proliferative activity of the cells following treatment with the test agents was examined by ELISA, BrdU-colorimetric immunoassay [Cell Proliferation ELISA, BrdU (colorimetric); Roche Applied Science] according to the manufacturer's instructions. This method is based on the incorporation of the pyrimidine analogue-bromodeoxyuridine (BrdU)-instead of thymidine into the DNA of proliferating cells. The B16.F10 melanoma cells in the co-culture model were incubated with BrdU solution for 24 h and the culture medium was completely removed from each well. Following this step, the cells were fixed and

the DNA was denatured. A monoclonal antibody conjugated with peroxidase (anti-BrdU-POD, cat. no. 11647229001, Roche Applied Science, dilution, 1:100; part of Cell Proliferation ELISA, BrdU kit) was added in each well, in order to detect the incorporated BrdU in the newly synthesized cellular DNA. The antibody was removed after 1 h of incubation at room temperature, and the cells were washed 3 times with phosphate-buffered saline. A peroxidase substrate (tetramethyl-benzidine) was added to each well, and the immune complexes were detected by measuring the absorbance of the reaction product at 450 nm with a reference wavelength of 655 nm using a microplate reader (BMG Labtech, serial no. 415-1324), as previously described (38).

Assessment of apoptosis/necrosis in the co-culture model. To determine the capacity of the combination therapy to induce the apoptosis of melanoma cells co-cultured with murine macrophages, we used the Annexin V-fluorescein isothiocyanate (FITC) assay (Cayman Chemical Co.). The principle of this protocol is based on the externalization of phosphatidylserine and phosphatidylethanolamine on the outer leaflet of the plasma membrane of the apoptotic cells. The redistribution of the phospholipids is measured after high-affinity binding to Annexin V conjugated with FITC. Thus, $5x10^4$ cells/well ($1x10^4$ melanoma cells and $4x10^4$ macrophages) were seeded in a 96-well black culture plate for 24 h. Several concentrations of DOX were added in triplicate at concentrations ranging from 0.03 to 0.5 μM. PLP was used at the concentration of 410 μM based on our previous reported data showing that this is the maximum concentration that could be achieved in tumors (37). At the end of the incubation period, a double staining with Annexin V-FITC and propidium iodide (PI) was performed at room temperature for 10 min as described previously (39). Fluorescence was determined using a fluorescence plate reader (BMG Labtech, serial no. 415-1324). The fluorescence emitted by early apoptotic cells (Annexin V $FITC^+/PI^-$) was measured at 535 nm with an excitation wavelength of 485 nm, whereas fluorescence determined by late apoptotic and necrotic cells was measured at 640 nm with excitation at 540 nm. 5-Fluorouracil (Sigma-Aldrich) (25 mg/ml)-treated cells were used as a positive control for late apoptosis. Cells cultured only in cell culture medium were used as a negative control, as previously described (39).

Preparation of cell lysates. To obtain cell lysates, melanoma cells co-cultured with macrophages, as well as IL-4-polarized macrophages following 24 h of incubation with the different treatments, were washed with PBS and viable (adherent) cells were mechanically detached and lysed with cell lysis buffer (10 mM Hepes, 200 mM NaCl, 1% Triton X, 10 mM $MgCl_2$, 1 mM DTT), following 30 min of incubation on ice. Complete Protease Inhibitor Cocktail tablets (Roche Applied Science) were added to the lysis buffer. Cell lysates were cleared by centrifugation at 18,000 x g for 10 min at 4˚C and the supernatant was collected. The protein content of the cell lysates was determined by Bradford assay (Sigma-Aldrich).

HPLC determination of malondialdehyde levels in cell co-culture. Malondialdehyde (MDA) is the main product of lipid peroxidation mediated by reactive oxygen species (ROS) and therefore, it is a good indicator of the overall levels of oxidative stress. MDA levels in the cell lysates were determined according to the method employed by Karatas *et al* (2002) (40) through high-performance liquid chromatography (HPLC) as previously described (38). The column type was RP18 (5 μm) (Supelco) and the mobile phase consisted of 30 mM KH_2PO_4/methanol in a volume ratio of 65:35. The flow rate was set at 0.5 ml/min and MDA was measured using a UV detector (Jasco Corp.) set at 254 nm. Each sample was tested in duplicate. The retention time of MDA was approximately 5.4 min. Data were expressed as μmoles of MDA/mg of protein.

Angiogenic and inflammatory protein array. To assess whether the combination therapy alters the cell production of proteins involved in angiogenesis and inflammation, a screening for 24 proteins involved in angiogenesis and inflammation was performed as previously described by using a protein array of RayBio® Mouse Angiogenic protein Antibody Array membranes 1.1 (RayBiotech Inc.) (39). One array membrane containing 24 types of primary antibodies against specific mouse proteins was used per cell lysate. The array membranes were incubated with 200 μg of proteins of cell lysates, overnight at 4˚C. A mixture of secondary biotin-conjugated antibodies against the same angiogenic proteins as those for primary antibodies (included in the array), was added on the membranes and incubated for 2 h at room temperature, followed by incubation with HRP-conjugated streptavidin for an additional 2 h at room temperature. Each incubation step was followed by 5 washing steps. Thereafter, the membranes were incubated with a mixture of two detection buffers for 1 min, exposed to an X-ray film (Kodak) for 4 min and then the films were developed. The protein expression level was quantified by measuring the intensity of the color of each spot on the membranes, in comparison to the positive control spots already bound to the membranes, using TotalLab Quant Software version 12 for Windows. Each protein level from the treated groups was expressed as percentage of the same protein level from the untreated cells (controls). Each protein for each experimental group was determined in duplicate.

RT-qPCR quantification of markers for the IL-4 polarization of macrophages. Total RNA was isolated from the M2 polarized murine macrophages following treatment with either 410 μM PLP + 0.06 μM DOX or 410 μM PLP + 0.5 μM DOX using a RNA kit (peqGOLD Total RNA kit, PeqLab). Untreated cells were used as a control. To avoid contamination with genomic DNA, 1 μg of total RNA was digested with 1U of RNase free DNase (Thermo Fisher Scientific, Inc.) for 30 min at 37˚C followed by the addition of EDTA and incubation at 65˚C for 10 min. Following DNase digestion, 750 ng of total RNA were reverse-transcribed into cDNA using the Verso cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Identical samples from each experimental group were processed in the absence of reverse transcriptase and served as controls for genomic DNA contamination. Reverse transcription products (1 μl) were amplified in a 25-μl reaction mix containing 1X Maxima SYBR-Green qPCR Master Mix (Thermo Fisher Scientific, Inc.), and 0.3 μM of each primer using a Corbett

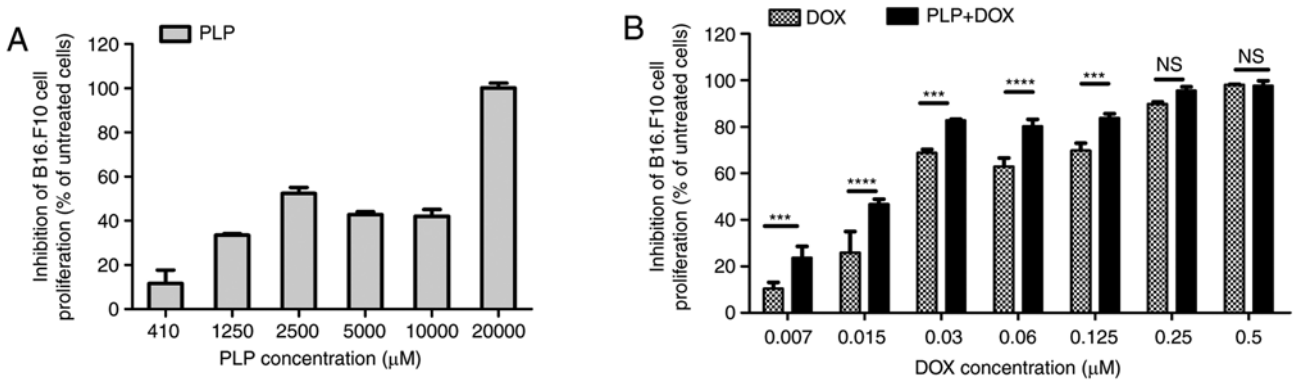

Figure 1. Effects of different treatments on B16.F10 cell proliferation. (A) B16.F10 mouse melanoma cells co-cultured with murine macrophages were incubated with various concentrations of PLP 410-20,000 μM for 24 h. (B) B16F10 mouse melanoma cells co-cultured with murine macrophages were incubated with various concentrations of DOX ranging from 0.007-0.5 μM in the presence, as well as in the absence of 410 μM PLP for 24 h. Data are shown as the means ± SD of 3 measurements and represented as percentages of proliferation inhibition compared with the proliferation of control cells. Two-way ANOVA followed by the Bonferroni post hoc test was used to assess statistical differences between the anti-proliferative effects of DOX administered alone, as well as in combination with PLP (NS, not significant, P>0.05; ***P<0.001; ****P<0.0001). PLP, cells incubated with various concentrations of prednisolone disodium phosphate; DOX, cells incubated with various concentrations of doxorubicin; PLP + DOX, cells incubated with various concentrations of DOX and 410 μM PLP.

RotorGene instrument using the following cycling parameters: Pre-incubation at 95˚C for 10 min, then cycling: 95˚C for 15 sec, 60˚C for 30 sec, and then 72˚C for 30 sec. To examine for primer specificity, melting curves were generated.

The sequences of the primers were as follows: Mouse IL-10 forward, 5'-GGTTGCCAAGCCTTATCGGA-3' and reverse, 5'-ACCTGCTCCACTGCCTTGCT-3'; mouse arginase-1 (Arg-1) forward, 5'-CTCCAAGCCAAAGTCCTTAGAG-3' and reverse, 5'-AGGAGCTGTCATTAGGGACATC-3; and mouse β-actin forward, 5'-TCTTTGCAGCTCCTTCGTTGCCGG TCC-3' and reverse, 5'-GTCCTTCTGACCCATTCCCACCAT CACAC-3'. Gene expression was calculated by relative quantitation using the comparative Cq method (ΔΔCq), as previously described (41). Mouse β-actin was used as a reference gene. Gene expression was reported as fold change ($2^{-\Delta\Delta Ct}$), relative to the untreated control cells, used as a calibrator.

Statistical analysis. Data from different experiments are reported as the means ± standard deviation (SD). The IC_{50} values of different treatments were calculated using non-linear regression of sigmoidal dose response curves offered by the GraphPad Prism version 6 for Windows (GraphPad Software, Inc.). The differences between the effects of treatments on the production of markers for specific pro-tumor processes were analyzed by one-way ANOVA. To analyze the treatment effects on cell proliferation, as well as on the levels of angiogenic/inflammatory proteins in cells, two-way ANOVA with Bonferroni correction for multiple comparisons was used. All statistical analyses were performed using the same statistical software mentioned above. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxic effects of the combination therapy. To investigate whether PLP can enhance the antitumor effects of DOX on B16.F10 melanoma cells, various concentrations of DOX were administered alone, as well as in combination with PLP in the co-culture model. The cytotoxic effects of the various treatments on the B16.F0 melanoma cells co-cultured with macrophages were assessed with regard to cancer cell proliferation (Fig. 1 and Table I) and the induction of apoptosis in the cell co-culture (Fig. 2).

Synergistic effects of combined treatment with PLP and DOX on B16.F10 cell proliferation. The effects of these treatments on cancer cell proliferation were expressed as percentages of the inhibition of cell proliferation compared to the proliferation of the B16.F10 cells used as controls (Fig. 1A and B). The IC_{50} values for PLP and DOX administered as single treatments, as well as in combination, on B16.F10 cell proliferation are shown in Table I.

In line with our previous study regarding the cytotoxicity of PLP on cancer cells (28), 410 μM PLP was the first concentration of glucocorticoid that exerted slight to moderate inhibitory effects on melanoma cell proliferation. This concentration was selected to be administered in combination with the cytotoxic drug, DOX in the cell co-culture model. Apart from the highest concentrations of DOX (0.25 μM and 0.5 μM), combined treatment with 410 μM PLP with each DOX concentration significantly enhanced the anti-proliferative effects of the cytotoxic drug on the tumor cells (Fig. 1B). As at the concentrations of 0.25 μM and 0.5 μM DOX, the proliferation of B16.F10 cells was completely compromised, the enhancing effects of PLP on the DOX cytotoxicity on these cancer cells were overshadowed. Moreover, the IC_{50} value of DOX decreased 2-fold when the cytotoxic drug treatment was administered in combination with 410 μM PLP (Table I). Thus, according to the Chou-Talalay calculation method, the combination index (CI) (42,43) indicated synergism between the anti-proliferative effects of PLP and DOX on melanoma cells (CI=0.668) (Table I). Since 0.06 μM was the lowest concentration of DOX which, when used in combination with 410 μM PLP exerted a potent inhibitory effect (by 80% inhibition of B16.F10 cell proliferation) (Fig. 1B) on cancer cell proliferation, this combination was used throughout the experiments to further investigate the molecular mechanisms responsible for the synergistic antitumor efficacy of both drugs on melanoma microenvironment model *in vitro*.

Table I. Synergistic effect of co-treatment with PLP and DOX on B16.F10 cell proliferation.

Treatment	IC_{50} (μM)	95% confidence interval	Combination index (CI)	
			CI value	Interpretation
DOX	0.029	0.023 to 0.040	-	-
PLP	2706.0	911.4 to 8035	-	-
410 μM PLP + DOX	0.015	0.013 to 0.018	0.668	Synergism

IC_{50} represents the half maximal inhibitory concentration for the tested drugs and CI represents the 'combination index', which quantitatively depicts synergism (CI<1), additive effect (CI=1), and antagonism (CI>1), according to the Chou-Talalay method (41,42).

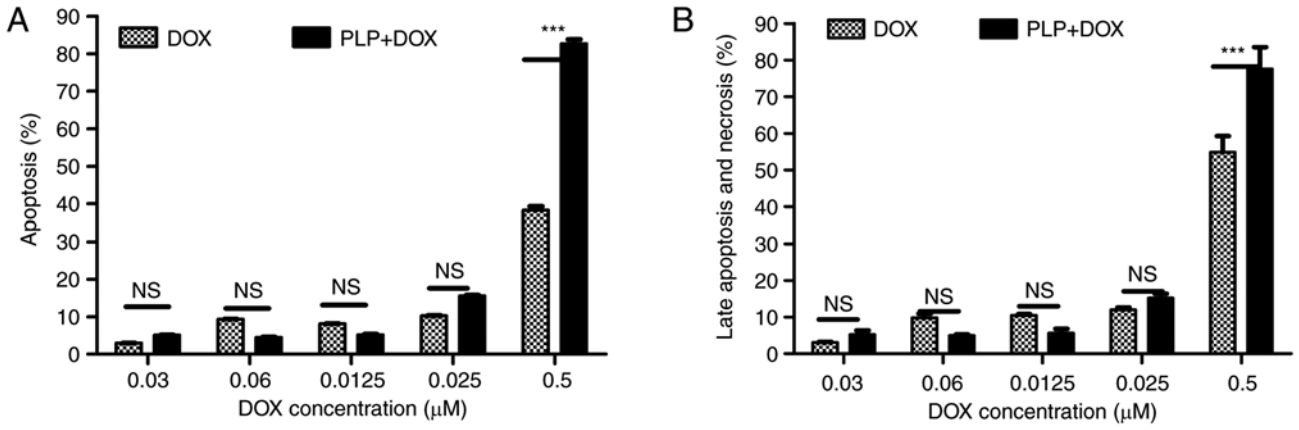

Figure 2. Evaluation of the ability of PLP + DOX to induce cell apoptosis and necrosis. B16.F10 mouse melanoma cells co-cultivated with murine macrophages were incubated with various concentrations of DOX ranging from 0.03-0.5 μM in the presence, as well as in the absence of 410 μM PLP for 24 h. A double labeling with Annexin V-FITC and PI was used to assess early apoptosis and necrosis, respectively. Relative fluorescence units measured were normalized for the number of cells under each condition. Data are shown as percentages of (A) apoptosis and (B) late apoptosis and necrosis in comparison with positive controls and represented as mean ± SD of 3 measurements. Two-way ANOVA followed by the Bonferroni post hoc test was used to assess statistically significant differences between the effects of DOX administered alone, as well as in combination with PLP (NS, not significant, $P>0.05$; $^{***}P<0.001$). DOX, cells incubated with various concentrations of doxorubicin; PLP + DOX, cells incubated with various concentrations of DOX and 410 μM prednisolone disodium phosphate.

Apoptotic and necrotic effects of the combined treatment. To gain insights into the mechanisms through which the combination therapy inhibited tumor cell proliferation, we assessed the ability of the treatment to induce the apoptosis of B16.F10 murine melanoma cells co-cultured with TAMs. Thus, we used Annexin V-FITC to stain the cells in early apoptosis and PI for late apoptosis and necrosis. Relative fluorescence units measured were normalized for the number of cells. Subsequently, all data were compared with the positive control and shown as percentages of apoptosis (Fig. 2A) and as percentages of necrosis and late apoptosis (Fig. 2B).

Following 24 h of incubation with DOX alone, moderate apoptotic (approximately 40%) (Fig. 2A) and necrotic effects (approximately 50%) (Fig. 2B) were noted only at the highest concentration tested. Notably, the same concentration of DOX administered in combination with 410 μM PLP induced potent apoptotic (approximately 80%) (Fig. 2A) and necrotic effects (approximately 80%) on the cell co-culture (Fig. 2B).

Therefore, this combination treatment (410 μM PLP + 0.5 μM DOX) with potent apoptotic and necrotic effects on the cell co-culture was also selected to be investigated with regard to the underlying mechanisms of the cytotoxicity of both drugs on melanoma microenvironment *in vitro*.

Effect of the combined treatments on intracellular oxidative stress. As melanoma cells are under persistent oxidative stress levels (44), we evaluated whether each combined treatment could affect the physiological production of ROS in murine melanoma cells cultured with TAMs. Therefore, the levels of MDA, a general marker of oxidative stress, in the cell co-culture lysates were assessed and are shown in Fig. 3. Our data revealed that treatment with 0.06 μM DOX either alone or in combination with PLP markedly inhibited (by ~70%) the production of MDA in the B16.F10 melanoma cells co-cultured with murine macrophages (Fig. 3), while treatment with 410 μM PLP alone had no effect on intracellular oxidative stress. Nevertheless, the treatment with the pro-apoptotic concentration of DOX (0.5 μM) alone, as well as in the presence of PLP, did not induce any significant modification of the intracellular oxidative stress in the cancer cells co-cultured with TAMs (Fig. 3).

Effects of the combined treatments on the angiogenic/inflammatory capacity of B16.F10 cells co-cultured with TAMs. The effects of the different treatments on the expression levels of the angiogenic/inflammatory proteins in B16.F10 cells co-cultured with murine macrophages were evaluated

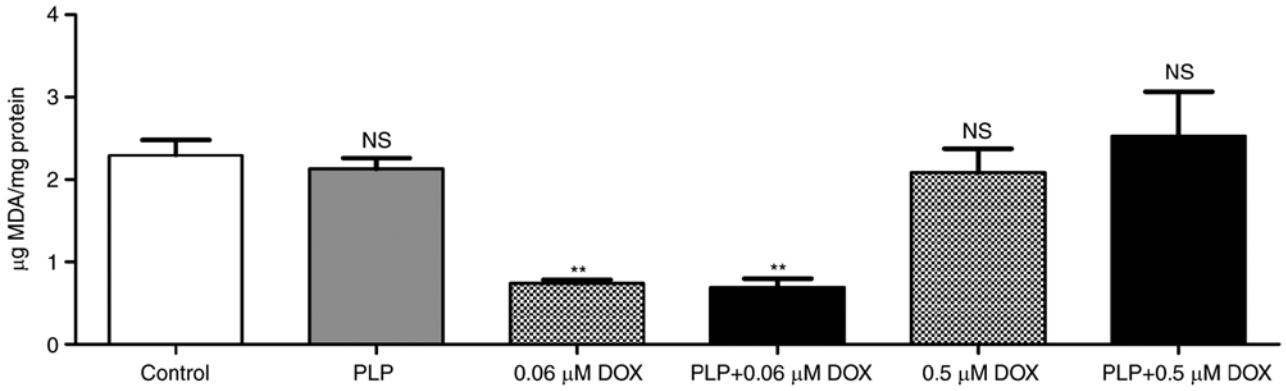

Figure 3. Evaluation of the effect of PLP + DOX on oxidative stress in B16.F10 melanoma cells co-cultured with murine macrophages. The level of MDA was determined by HPLC analysis. Control, MDA levels in untreated cells following 24 h of incubation with culture media; 0.06 μM DOX, MDA levels in cells afte following r 24 h of incubation with 0.06 μM DOX; PLP, MDA levels in cells following 24 h of incubation with medium supplemented with 410 μM PLP; PLP + 0.06 μM DOX, MDA levels in cells following 24 h of treatment with 0.06 μM DOX + 410 μM PLP; 0.5 μM DOX, MDA levels in cells following 24 h of incubation with 0.5 μM DOX; PLP + 0.5 μM DOX, MDA levels in cells following 24 h of treatment with 0.5 μM DOX + 410 μM PLP. The results are expressed as the means ± SD of 2 independent measurements. One-way ANOVA with the Bonferroni post hoc test was performed to analyze the differences between the MDA levels in the control cells and treated cells (NS, not significant, $P>0.05$; $^{**}P<0.01$). MDA, malondialdehyde; PLP, prednisolone disodium phosphate; DOX, doxorubicin.

by performing a screening for 24 angiogenic and inflammatory proteins using a protein array (RayBiotech Inc.) and results are shown in Fig. 4 and Table II. In accordance with our previously published data (26), the overall production of angiogenic/inflammatory proteins was notably decreased by 40% ($P<0.001$) in the cells treated with 410 μM PLP compared to their production in the untreated cell co-culture, as a result of the well-known anti-inflammatory effects of this drug. The addition of 0.06 μM DOX to the co-culture did not lead to a significant decrease in the mean production of the angiogenic/inflammatory proteins compared with cells treated with PLP alone. More specifically, 0.06 μM DOX stimulated the production of two proteins with an important role in tumor progression: Insulin-like growth factor (IGF)-II (by 45%) and IL-1 β (by 94%), while the levels of the antitumor protein, tissue inhibitor of metalloproteinases (TIMP)-1, were markedly decreased (by 80%). Only the production of tumorigenic proteins, such as granulocyte-colony stimulating factor (G-CSF), M-CSF, IL-6, basic fibroblast growth factor (bFGF) and IL-13 was moderately decreased (by 35-55%) (Fig. 4).

Notably, a marked overall reduction by 52% ($P<0.001$) of angiogenic protein production in the cell co-culture lysates was observed following combined treatment (410 μM PLP + 0.06 μM DOX) with anti-proliferative effects, while the reduction following co-treatment with both drugs at concentrations with pro-apoptotic, as well as anti-proliferative effects (410 μM PLP + 0.5 μM DOX) was slightly higher (by 66%, $P<0.001$). More specifically, the production of the majority of the pro-angiogenic proteins was reduced almost completely (70-100% reduction in G-CSF, IL-1α, IL-1β, IL-9, IL-12p40, FasL, bFGF, leptin, TIMP-1 and TIMP-2) following treatment with 410 μM PLP + 0.5 μM DOX. Nevertheless, the levels of antitumor proteins [platelet factor (PF)-4, IL-12p70, IFN-γ and monokine induced by IFN-γ (MIG)] were also moderately to strongly decreased following treatment with each of the combination treatments tested (Table II).

Combined treatments affect the pro-angiogenic functions of TAMs. As TAMs are key cell players in the tumor angiogenesis, we investigated whether the anti-angiogenic effects of the combined treatments on the cell co-culture model could be linked to their effects on TAMs angiogenic capacity. Thus, IL-4 polarized macrophages were treated with 410 μM PLP + 0.06 μM DOX, as well as 410 μM PLP + 0.5 μM DOX. Following 24 h of incubation with 410 μM PLP + 0.06 μM DOX, the average angiogenic protein production in the polarized macrophages was not affected. Only the levels of specific pro-angiogenic proteins (GM-CSF, IL-1β, TNF-α, eotaxin, FasL, bFGF and VEGF) were moderately (by approximately 40-50%) decreased by this combined treatment. Notably, combined treatment with both drugs at concentrations demonstrating pro-apoptotic, as well as anti-proliferative effects (410 μM PLP + 0.5 μM DOX) markedly inhibited the overall expression of the angiogenic proteins (by approximately 70%, $P<0.001$). In particular, treatment with 410 μM PLP + 0.5 μM DOX decreased the production of the majority of the tumorigenic proteins (55-100% reduction in G-CSF, GM-CSF, M-CSF, IGF-II, IL-1α, IL-1β, IL-6, IL-9, IL-12p40, IL-13, MCP-1, eotaxin, FasL, bFGF, leptin and TIMP-2) (Table III), as well as that of the anti-angiogenic proteins (55-95% reduction in PF-4, IL-12p70, IFN-γ and MIG) (Table III).

Combined treatment does not affect the polarization of TAMs. To assess whether the applied treatments can affect the polarization of TAMs, the mRNA relative expression of two markers for this protumor phenotype of macrophages (IL-10 and Arg-1) (45) was quantified by RT-qPCR. The results revealed that administration of each combined treatment increased the expression of both markers, albeit with lower and not statistically significant degrees for pro-apoptotic combined treatment (Fig. 5).

Previously reported data have demonstrated that TAMs that induce chemoresistance to DOX are characterized by a high expression of CD68, CD206, CD163, programmed death-ligand 1 (PD-L1) and release immunosuppressive cytokines, such as

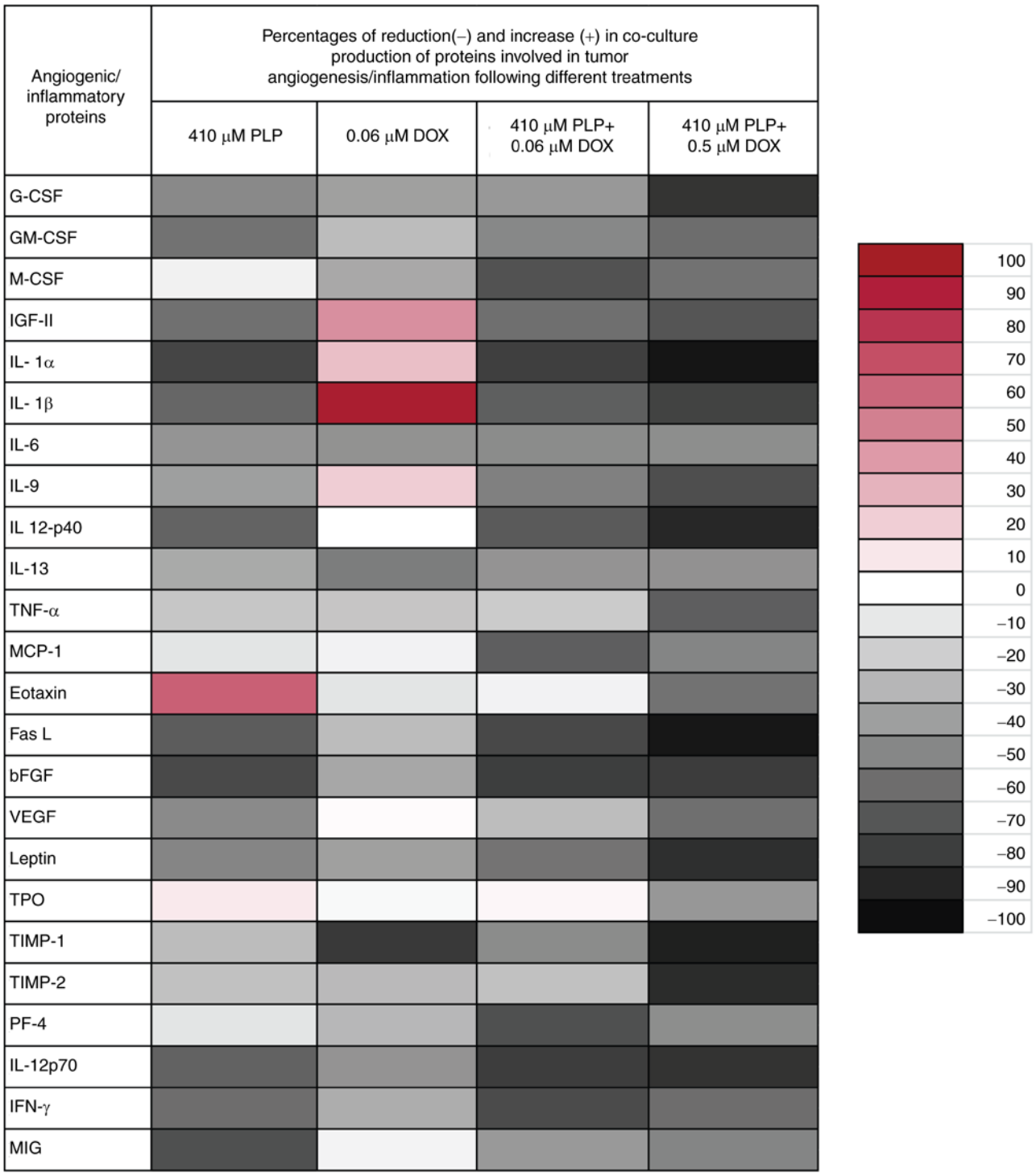

Figure 4. Effects of PLP, DOX and PLP + DOX on the production of angiogenic/inflammatory proteins in the co-culture of B16F10 melanoma cells and murine macrophages. Results are presented either as % of reduction (-) of tumor protein levels ranging from 0% (white) to 100% (black with vertical line pattern) or as % of stimulation (+) of production of proteins ranging from 0% (white) to 100% (red) in cells after different treatments compared to levels of the same proteins in untreated cells. G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage-colony stimulating factor; M-CSF, monocyte-colony stimulating factor; IGF-II, insulin growth factor II; IL, interleukin; TNF-α, tumor necrosis factor α; MCP-1, monocyte chemoattractant protein-1; FasL, Fas ligand; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; TPO, thrombopoietin; TIMP, tissue inhibitor of matrix metalloproteinase; PF-4, platelet factor 4; IFN-γ, interferon γ; MIG, monokine induced by IFN-γ.

IL-10 and TGF-β (46). Moreover, in mouse models for breast and lung cancer, the administration of cyclophosphamide, paclitaxel and DOX, was shown to promote the protumor phenotype of TAMs (46). Thus, the strong overexpression of both M2 macrophage markers induced by the anti-proliferative dose (0.06 μM) of DOX suggest that a low dose of the cytotoxic drug favors the pro-tumor and immunosuppressive function of TAMs. Thus, it is suggested that the combined treatments tested did not affect the M2 phenotype of macrophages and only had the ability to suppress the pro-angiogenic functions of this cell type.

Table II. Effects of PLP + DOX on the production of angiogenic/inflammatory proteins in B16.F10 murine melanoma cells co-cultured with murine macrophages for 24 h.

Angiogenic/inflammatory proteins	Percentage of reduction(-)/increase (+) in the cell co-culture production of proteins involved in tumor angiogenesis/inflammation following different combined treatments compared to untreated cells		
	410 μM PLP	410 μM PLP + 0.06 μM DOX	410 μM PLP + 0.5 μM DOX
G-CSF	-49.11±9.01[c]	-42.45±4.76[c]	-79.72±0.88[c,f]
GM-CSF	-58.61±0.40[c]	-49.31±8.34[c]	-57.40±4.61[c,d]
M-CSF	-6.69±32.26[a]	-71.58±2.87[c]	-55.53±4.90[c,d]
IGF-II	-59.21±2.12[c]	-59.56±0.20[c]	-66.83±2.02[c,d]
IL-1α	-77.01±9.54[c]	-79.39±3.95[c]	-91.38±0.09[c,d]
IL-1β	-63.39±1.52[c]	-66.30±0.61[c]	-74.37±0.54[c,d]
IL-6	-44.62±1.79[c]	-48.03±4.21[c]	-45.05±12.33[c,d]
IL-9	-39.75±3.19[b]	-52.90±2.24[c]	-69.65±3.09[c,e]
IL 12-p40	-64.75±1.24[c]	-68.24±1.48[c]	-84.69±3.30[c,d]
IL-13	-35.42±3.14[b]	-44.66±0.27[c]	-43.26±10.9[c,d]
TNF-α	-23.34±1.04[a]	-21.19±0.71[a]	-63.20±1.70[a,f]
MCP-1	11.16±10.13[a]	-66.69±0.67[c]	-48.53±1.50[c,e]
Eotaxin	62.79±24.13[c]	-6.31±4.64[a]	-55.66±15.11[c,f]
FasL	-67.41±1.89[c]	-75.96±0.85[c]	-90.21±1.09[c,d]
bFGF	-75.46±0.48[c]	-80.16±1.60[c]	-76.77±1.22[c,d]
VEGF	-48.35±1.93[c]	-26.86±16.64[a]	-56.74±0.91[c,f]
Leptin	-50.62±2.14[c]	-57.91±5.01[c]	-81.72±2.40[c,f]
TPO	9.69±3.79[a]	+4.51±5.67[a]	-41.10±0.02[c,f]
TIMP-1	-27.53±7.18[a]	-47.98±0.72[c]	-87.82±1.52[c,f]
TIMP-2	25.23±13.87[a]	-25.50±5.15[a]	-82.97±0.27[c,f]
PF4	-11.36±6.98[a]	-72.45±4.64[c]	-44.83±0.14[c,f]
IL-12p70	-65.28±2.69[c]	-80.26±0.96[c]	-80.09±0.96[c,d]
IFN-γ	-60.24±2.70[c]	-74.73±2.69[c]	-57.13±0.17[c,e]
MIG	-72.60±2.29[c]	-41.83±7.01[c]	-48.43±8.03[c,d]

The results represent the means ± SD 2 two independent measurements. 410 μM PLP + 0.06 μM DOX, percentages of reduction or increase in different protein production in cells incubated with 410 μM PLP + 0.06 μM DOX for 24 h compared with their production in untreated cells; 410 μM PLP + 0.5 μM DOX, percentages of reduction or increase in different protein production in cells incubated with 410 μM PLP + 0.5 μM DOX for 24 h compared with their production in untreated cells. P-value were determined to evaluate the statistical significance of the data and were calculated by two-way ANOVA with the Bonferroni post hoc test ([a]P>0.05, not significant; [b]P<0.01; [c]P<0.001). The percentages of reduction or increase in different protein production in cells incubated with 410 μM PLP + 0.06 μM DOX were also compared with their production in cells treated with 410 μM PLP + 0.5 μM DOX; ([d]P>0.05, not significant; [e]P<0.05; [f]P<0.001).

Discussion

Regardless of recent advances in melanoma treatment, the development of acquired drug resistance remains a major issue, limiting the effectiveness of these therapies (9,47). One of the principal causes for melanoma cell resistance to different treatments is the involvement of the tumor microenvironment cell types (49,50). Among these supportive cell types, TAMs are the most abundant immune cells infiltrated in the stroma of solid cancers, their elevated number being an indicate of a poor prognosis for >80% of tumor cases. Intratumor macrophages exhibit a high plasticity in response to microenvironment stimuli, promoting immune suppression, tumor cell proliferation, angiogenesis, invasion and metastasis (51). In particular, it has been demonstrated that TAMs may interfere via different means with the response of melanoma cells to various drugs (52-55). Nevertheless, our previous studies demonstrated that melanoma growth can be markedly inhibited following the administration of the water-soluble salt of prednisolone (PLP; as a liposomal formulation), as a result of its inhibitory effect on TAM-mediated tumor angiogenesis (26,56). Based on these findings, the aim of the present study was to investigate whether the combination of the TAM-targeting drug, PLP (26), with a conventional cytotoxic drug, DOX, could lead to an improved therapeutic outcome on the melanoma microenvironment. To the best of our knowledge, the cytotoxicity of this combined therapeutic approach on the melanoma microenvironment model has not been described to date. The results of this study provided confirmatory evidence for the synergistic inhibitory effects of PLP and DOX on the

Table III. Effects of the combined treatments on the production of angiogenic/inflammatory proteins in IL-4 polarized murine macrophages.

Angiogenic/ inflammatory proteins	Percentage of reduction (-)/increase (+) in IL-4 polarized macrophages production of proteins involved in tumor angiogenesis/inflammation following the treatment with DOX and PLP compared to untreated macrophages	
	410 μM PLP + 0.06 μM DOX	410 μM PLP + 0.5 μM DOX
G-CSF	-16.58±4.38[a]	-69.06±3.33[d,h]
GM-CSF	-40.67±3.52[c]	-87.93±0.54[d,h]
M-CSF	17.62±7.15[a]	-75.82±3.80[d,h]
IGF-II	65.93±29.00[d]	-80.63±0.9[d,h]
IL-1α	38.64±2.20[b]	-84.63±2.03[d,h]
IL-1β	-44.84±7.51[c]	-60.25±5.71[d,e]
IL-6	-28.59±2.57[a]	-80.58±9.13[d,h]
IL-9	-1.98±7.55[a]	-84.80±3.72[d,h]
IL 12-p40	-13.26±0.94[a]	-92.62±0.09[d,h]
IL-13	-10.11±4.65[a]	-70.03±3.07[d,h]
TNF-α	-45.07±9.47[c]	-55.91±1.91[d,g]
MCP-1	+26.15±13.48[a]	-81.71±0.34[d,f]
Eotaxin	-42.71±8.93[c]	-88.89±1.36[d,h]
FasL	-47.74±2.21[d]	-94.03±0.82[d,h]
bFGF	-50.00±1.71[d]	-77.00±5.04[b,e]
VEGF	-51.06±0.58[d]	-34.30±5.23[b,e]
Leptin	-32.70±11.94[a]	-98.77±0[d,h]
TPO	-25.24±11.43[a]	-40.95±4.81[c,e]
TIMP-1	-1.12±3.71[a]	22.93±2.18[a,e]
TIMP-2	-12.87±2.54[a]	-89.81±5.81[d,h]
PF-4	11.18±27.46[a]	-54.42±4.40[d,g]
IL-12p70	-3.16±0.72[a]	-79.50±1.42[d,h]
IFN-γ	-8.50±11.00[a]	-92.25±2.22[d,h]
MIG	-29.17±16.36[a]	-93.32±1.69[d,h]

The results represent the means ± SD of 2 independent measurements. 410 μM PLP + 0.06 μM DOX, percentages of reduction or increase in different protein production in cells incubated with 410 μM PLP + 0.06 μM DOX for 24 h compared with their production in untreated cells; 410 μM PLP + 0.5 μM DOX, percentages of reduction or increase in different protein production in cells incubated with 410 μM PLP + 0.5 μM DOX for 24 h compared with their production in untreated cells. P-values were determined to evaluate the statistical significance of the data and were calculated by two-way ANOVA with the Bonferroni post hoc test ([a]P>0.05, not significant; [b]P<0.05; [c]P<0.01; [d]P<0.001). The percentages of reduction or increase in different protein production in cells incubated with 410 μM PLP + 0.06 μM DOX were also compared with their production in cells treated with 410 μM PLP + 0.5 μM DOX; ([e]P>0.05, not significant; [f]P<0.05; [g]P<0.01; [h]P<0.001).

proliferation of B16.F10 melanoma cells (Fig. 1 and Table I). Moreover, the pro-apoptotic effects of DOX (noted at the highest concentration tested) on the melanoma microenvironment were also significantly potentiated by glucocorticoid administration (Fig. 2). To elucidate the molecular mechanisms responsible for the potent cytotoxicity of the combined therapeutic approach on B16.F10 melanoma cells, we explored the effects of two different combined treatments based on 410 μM PLP administration with either the anti-proliferative DOX concentration (0.06 μM) or the pro-apoptotic concentration of DOX (0.5 μM), on TAM-mediated protumor processes, such as oxidative stress and angiogenesis.

As several lines of evidence have demonstrated the pro-oxidant capacity of DOX in both normal and cancer cells (57,58) the role of the modulation of oxidative stress in the cytotoxicity of the combined administration of PLP and DOX on B16.F10 cells co-cultured with TAMs was assessed. The results suggested that only the administration of 0.06 μM DOX exerted potent antioxidant effects on the melanoma microenvironment, irrespective of the presence of PLP (Fig. 3). Although the majority of studies have indicated the stimulatory effects of DOX on oxidative stress in endothelial and myocardial cells (59,60), the suppressive effects of a lower concentration of DOX on ROS levels in the melanoma microenvironment may be explained by its potential to activate the antioxidant enzymes, as previously suggested (61,62). Taken together, these data suggested that the modulation of intratumor oxidative stress by this therapeutic approach may be responsible for the anti-proliferative activity rather than the inducing action of the combined administration of DOX and PLP on cell apoptosis. Moreover, it has been demonstrated that DOX-induced oxidative stress is mainly responsible for apoptosis in endothelial and myocardial cells, while tumor cell apoptosis is mediated via a different mechanism (59,60).

To link the cytotoxicity of the therapeutic approach to its ability to inhibit melanoma angiogenesis, we investigated whether the combined treatments affected the production of proteins involved in angiogenesis, as well as in inflammation-associated angiogenesis (Table II and Fig. 4). The data revealed that both combined treatments considerably decreased the levels of the majority of the pro-angiogenic/pro-inflammatory proteins in the cell co-culture (Table II and Fig. 4). These antitumor effects of the combined therapeutic approach may be linked to the anti-angiogenic activity of PLP on the melanoma microenvironment, as a single administration of the glucocorticoid also exerted marked inhibitory effects on the angiogenic capacity of the cell co-culture (Fig. 4). Notably, treatment with DOX alone did not markedly influence tumor angiogenesis (Fig. 4). These findings are consistent with the results of a previous study by our group, showing that the administration of DOX as a liposomal form, to melanoma tumor-bearing mice generated only a slight inhibitory effect on the production of angiogenic proteins (27). Taken together, these data suggest that the anti-angiogenic activity of PLP on the melanoma microenvironment may be potentiated by its combination with DOX in a cytotoxic drug concentration-dependent manner (Table II). Moreover, the higher amplitude of the anti-angiogenic effects of co-administration of PLP with 0.5 μM DOX than that induced by the administration of the same concentration of PLP in the presence of 0.06 μM DOX may also explain the pro-apoptotic effects of the first treatment. Thus, the production of several tumorigenic

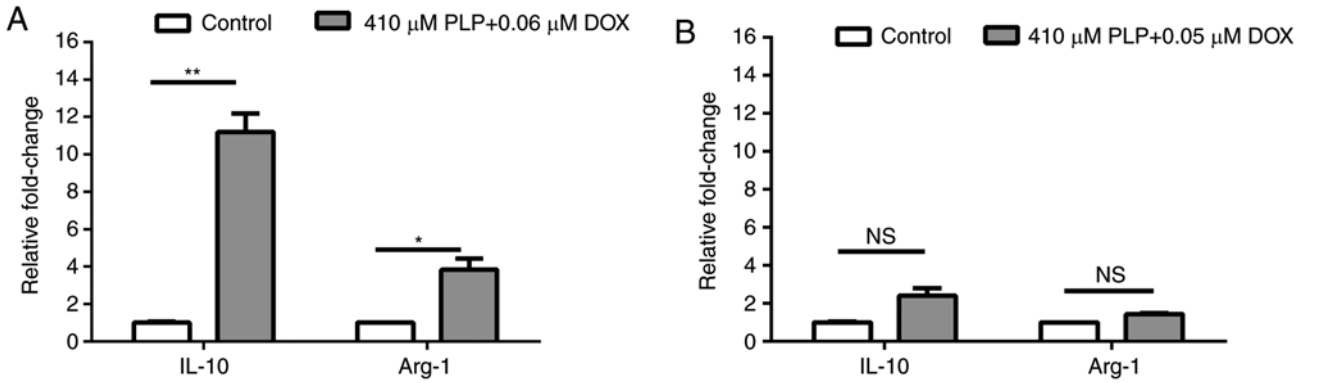

Figure 5. Effects of the combined treatments on the expression of IL-10 and Arg-1 in IL-4-polarized murine macrophages. (A) Relative fold induction of IL-10 and Arg-1 following treatment with 410 μM PLP + 0.06 μM DOX; (B) Relative fold induction of IL-10 and Arg-1 following the treatment with 410 μM PLP + 0.5 μM DOX; mRNA was quantified by RT-qPCR and the results are expressed as a fold change based on the Cq calculations. Untreated macrophages were used as calibrator (Control). P-values were determined to evaluate the statistical significance of the data and were calculated by one-way ANOVA with the Bonferroni post hoc test (NS, not significant, $P>0.05$; $^{*}P<0.05$; $^{**}P<0.01$).

proteins, such as TIMP-1, TIMP-2, TNF-α and thrombopoietin (TPO), also known for their anti-apoptotic activity (63-67), were markedly inhibited by the co-administration of PLP with 0.5 μM DOX.

Furthermore, to gain deeper insight into the role of TAMs in modulating melanoma angiogenesis and finally, in the response of melanoma cells to the combination therapy, the effects of the simultaneous administration of both drugs on the angiogenic capacity of TAMs were also evaluated. The results demonstrated differences in the underlying mechanisms of the anti-angiogenic action of the combined treatments on TAMs (Table III). Thus, the anti-proliferative DOX concentration (0.06 μM) administered in combination with PLP, exerted moderate suppressive effects on the TAM levels of specific pro-angiogenic proteins (GM-CSF, IL-1β, TNF-α, eotaxin, FasL, bFGF and VEGF), while the anti-angiogenic and anti-inflammatory proteins (PF-4, IL-12p70, IFN-γ and MIG) were not affected by this treatment. When the same concentration of PLP was administered in the presence of the pro-apoptotic concentration of DOX (0.5 μM), the majority of the pro-angiogenic, as well as anti-angiogenic protein levels in TAMs were almost completely decreased (Table III). Nevertheless, the M2 phenotype of TAMs was not altered by any treatment, as the expression of IL-10 and Arg-1 was increased following combined treatment with PLP and 0.06 μM DOX, and was not affected by pro-apoptotic combined treatment (Fig. 5).

Collectively, the data of the present study suggested that both combined treatments inhibited the pro-angiogenic function of TAMs in the melanoma microenvironment, while the immunosuppressive phenotype of these macrophages (45,68,69) was not affected by these treatments. Consequently, the combined therapeutic approach developed in the present study could be improved by supplementation with IL-12 or IFN-γ as re-polarizing agents of M2-like TAMs toward M1-like antitumor macrophages (70).

In conclusion, the results of this study demonstrated that PLP enhanced the antitumor effects of DOX on B16.F10 murine melanoma cells compared with the effects induced by the cytotoxic drug administered alone. The cytotoxicity of DOX was potentiated mainly via the anti-angiogenic activity of PLP in the melanoma microenvironment. Moreover, the amplitude of the cytotoxicity of the combined treatments might be linked to the degree of the suppression of the pro-angiogenic function of TAMs. Nevertheless, the immunosuppressive phenotype of TAMs was still preserved after co-administration of PLP with DOX. Therefore, further investigations with regard to the re-activation of TAMs to combat melanoma cells are required.

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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

EL and MB conceived and designed the study. EL, VFR, LL, LP and AS acquired the data, and performed the experiments and statistical analysis. EL and MB analyzed and interpreted the data. EL and MB drafted and edited the manuscript. All authors have given final approval of the version to be published.

Ethics approval and consent to participate

Experiments using laboratory mice were performed according to the European and national regulations and were approved by the Committee on the Ethics of Animal Experiments of the Babes-Bolyai University (registration no. 31444/27.03.2017).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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