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Lansoprazole induces sensitivity to suboptimal doses of paclitaxel in human melanoma

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ABSTRACT

Tumor acidity is now considered an important determinant of drug-resistance and tumor progression, and anti-acidic approaches, such as Proton Pump inhibitors (PPIs), have demonstrated promising anti-tumor and chemo-sensitizing efficacy. The main purpose of the present study was to evaluate the possible PPI-induced sensitization of human melanoma cells to Paclitaxel (PTX). Our results show that PTX and the PPI Lansoprazole (LAN) combination was extremely efficient against metastatic melanoma cells, as compared to the single treatments, both *in vitro* and *in vivo*. We also showed that acidity plays an important role on the anti-tumor activity of these drugs, being detrimental for PTX activity, while crucial for the synergistic effect of PTX following pretreatment with LAN, due to its nature of pro-drug needing protonation for a full activation. We obtained straightforward results in a human melanoma xenograft model combining well tolerated LAN doses with suboptimal and poorly toxic doses of PTX. With this study we provide a clear evidence that the PPI LAN may be included in new combined therapy of human melanoma together with low doses of PTX.

Introduction

Malignant melanoma is one of the most highly invasive tumors, and its mortality rates have been rapidly increasing above those of any other cancers in recent years [1,2].

It is responsible for the majority of skin cancer-related mortality [3]. Surgical resection and systemic chemotherapy are still the main therapeutic strategies [4]. However the prognosis remains poor, mainly because standard chemo-therapeutics are associated with significant toxicity and low efficacy rates, especially once the metastatic process has begun [5,6].

Emerging studies indicate that a key factor contributing to poor responsiveness to chemotherapy and development of tumors' drug resistance is the acidic extracellular microenvironment [7]. It is well known that cancer cells take up much more glucose than normal cells and mainly process it through aerobic glycolysis, the so-called "Warburg effect" [8]. Such an altered metabolic pattern associates with an increased production of metabolic acids causing a drop in extracellular pH and a reversed intra-extracellular pH gradient [9]. Tumor acidosis is also an important determinant of tumor progression, and tumor pH regulation is increasingly being considered as an attractive therapeutic target [7,10–12]. Among the pH-regulating proteins, proton pumps, such as the vacuolar type – ATPase (V-ATPase), play an important role in both drug-resistance and metastatic spread.

The proton pump inhibitors (PPIs) are weak base pro-drugs that easily penetrate cell membranes and concentrate in acidic compartments, where they are converted into sulfonamide forms, representing the active inhibitors [13]. It has been reported that PPIs are chemosensitizing [14,15] as well as cytotoxic [16–18] drugs, active against several human tumor cells, such as melanoma [14,17,18], B cell-lymphomas [16], pancreatic cancer [19], gastric carcinoma [20–23], Ewing sarcoma [24], osteosarcomas [15,25], and breast cancer [26]. Very encouraging results have also been obtained in preliminary clinical studies, both in pets [27,28], and in humans [15]. In the case of melanoma, the PPI esomeprazole (ESOM) has been shown to have an antineoplastic effect [17,18], and to sensitize tumor cell lines to the effects of cisplatin [14], both *in vitro* and *in vivo*. Particularly, the microenvironmental low pH has been shown to represent one of the major mechanism of resistance of melanoma to chemotherapeutics [29].

Drugs that target the mitotic spindle are among the most effective anticancer therapeutics currently in use. Taxanes, like Paclitaxel (PTX), are alkaloid esters originally obtained from crude extracts of bark of the Pacific yew *Taxus brevifolia* [30]. These substances induce microtubule stabilization, leading to the arrest of cell proliferation and apoptosis [31]. PTX is widely used in many types of cancers.

Abbreviations: PTX, paclitaxel; LAN, lansoprazole; PPI, proton pump inhibitor.

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It has been used in clinical trials for the treatment of melanoma since 1990 [32], and it is currently used as a second line option in patients with metastatic melanoma, including patients whose disease has progressed after previous chemotherapy sessions [4]. Since PTX alone is often associated with high resistance [33,34], it is usually administered in combination with carboplatin [35]. However, this combination adds hematological toxicity without significantly improving response or survival rates [36].

The role of acidity or pH deregulation in tumor resistance or scarce sensitivity to PTX has been poorly investigated. However, PTX cytotoxicity but not intracellular uptake has been reported to be negatively affected by an acidic extracellular microenvironment [37]. Moreover, PTX antitumor efficacy has been shown to be potentiated by the concomitant specific inhibition of the Na(+)/H(+) exchanger isoform 1 (NHE1), which is aberrantly active in tumors and involved in tumor reversed intra-extracellular pH gradient [38].

The main purpose of the present study was to evaluate the possible PPI-induced sensitization of human melanoma cells to PTX. First, the *in vitro* cytotoxic properties of these compounds both alone and in combined treatment (Me 30966 and Mel 501) were investigated in human metastatic melanoma cell lines. Then, the effect of the *in vivo* treatment of melanoma-bearing CB-17 SCID/SCID mice with a relatively low dose of PTX was compared with that obtained using Lansoprazole (LAN) pretreatment followed by the same dose of PTX. The results suggest that combination of well-tolerated pH modulators, such as PPIs, with PTX might represent a new strategy for the treatment of melanoma, both providing a real anti-tumor strategy and allowing the use of suboptimal doses of chemotherapeutics, with a consequent reduction of systemic toxicity.

Materials and methods

Chemicals and reagents

LAN was provided by Takeda and resuspended at 30 mM in DMSO. PTX semisynthetic form, 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxymrthyl ester (BCECF, AM) and FITC-Dextran (average molecular weight 40.000) were purchased from Sigma-Aldrich (Milano, Italy) and resuspended in DMSO. PTX (Taxol®) for injection (30 mg/5 ml) was purchased from Bristol-Myers Squibb (Anagni, Italy). RPMI 1640 (BE12-702F), antibiotics (DE17-603E), phosphate buffer saline (PBS) (BE17-512F), trypsin/EDTA (BE17-171E) and fetal bovine serum (FBS) (DE14-701F) were from Lonza (Milano, Italy). Trypan blue was from Alexis Biochemicals (Firenze, Italy).

Cell lines

Metastatic melanoma cell lines Me 30966, Mel 501 (both supplied by Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics, at 37 °C in humidified 5% CO_2. Experiments were performed in unbuffered medium (without sodium bicarbonate), and in buffered medium (pH = 7.4).

Cell death determination

Melanoma cells were plated at $3–4 \times 10^4$ cells per well in 12-well plates in 1 ml of buffered RPMI medium. After 24 hours, the medium was replaced with fresh medium, buffered at pH = 7.4 or unbuffered. After 4 hours, necessary for the medium to acidify, cells were treated with doses 25, 50, 75, and 100 μM of LAN for 24 hours, or 5, 10 and 50 nM PTX for 48 hours. In combined treatment experiments, cells were pretreated for 24 hours with LAN 50 μM. The day after, the medium was replaced with fresh, buffered or unbuffered medium, and cells were then treated for an additional 48 hours with PTX 10 nM. After treatment, cells were collected by pooling cells from the medium (i.e., dead cells) and adherent (live) cells obtained by trypsinization. Cells were then centrifuged (10 minutes at 500 *g*), resuspended in PBS, and the suspension was diluted 1:1 (vol/vol) with 0.4% trypan blue. After 10 minutes cells were analyzed by FACS on a Becton Dickinson FACScalibur using CellQuestPro software (Becton Dickinson System). For each sample the total events were acquired in 60 seconds. All experiments were run in triplicate wells and repeated at least twice.

Cell proliferation

Melanoma cells were plated at 1×10^4 cells per well in 96-well plates in buffered RPMI medium. After 24 hours, the medium was replaced with fresh, buffered and unbuffered RPMI medium and cells were treated with LAN and/or PTX, as described for the determination of cell proliferation. After treatment, cell proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Milano, Italy). Cells were stained and analyzed on a Spectrophotometer ELx800 (Bio-Tek Instruments, Inc.). All experiments were run in triplicate wells and repeated at least twice.

Extracellular pH measurement

Extracellular pH (pHe) was measured according to Xiaodong Lu et al. [39]. Briefly, Me 30966 and Mel 501 cells were seeded in a 96 wells plate in buffered growth conditions (RPMI1640, 10% FCS). After 24 hours RPMI1640 was replaced by fresh, buffered and unbuffered media for 4 hours. After while, the medium was transferred into a 96 wells Nunc-Immuno MicroWell plate and in each well 3 μM BCECF was added for 20 min. Another set of experiment was performed to verify the extracellular pH after 24 hours of treatment with LAN. Me 30966 and Mel 501 cells were seeded in a 96 wells plate in buffered conditions (RPMI1640, 10% FCS). After 24 hours RPMI1640 was replaced by fresh, buffered and unbuffered media, and cells were treated with LAN 50 μM for 24 hours. After while, the medium was transferred into a 96 wells Nunc-Immuno MicroWell plate and in each well 3 μM BCECF was added for 20 min. For recovery experiments, cells were kept for a further 4 h in fresh, buffered and unbuffered media, following LAN treatment. Then, the medium was transferred into a 96 wells Nunc-Immuno MicroWell plate and in each well 3 μM BCECF was added for 20 min. Fluorescence intensity was measured with a fluorimeter Perkin-Elmer LS-50B, setting the instrument at the following wavelengths: 440 nm and 490 nm for excitation and 525 nm for emission. Standard curve was obtained by adding BCECF in RPMI 1640 plus 10% FCS, buffered at different pH (5.5–7.4). pHe was calculated utilizing 490/440 nm fluorescence ratio values and employing the standard curve and linear equation.

Vesicular pH measurement

Vesicular pH (pHves) was measured according to Ohkuma and Poole [40]. Briefly, Me 30966 and Mel 501 cells were seeded in a 96 wells Nunc-Immuno MicroWell plate in buffered growth conditions (RPMI1640, 10% FCS). After 24 hours RPMI1640 was replaced by fresh, buffered and unbuffered medium, and cells were treated with LAN 50 μM for 24 hours. FITC-Dextran was then added at a final concentration of 1 mg/ml for 4 hours. Fluorescence intensity was measured as described for extracellular pH measurement. Standard curve was obtained by adding FITC-Dextran in RPMI 1640 plus 10% FCS, buffered at different pH (4.0–6.1). pHves was calculated utilizing 490/440 nm fluorescence ratio values and employing the standard curve and linear equation.

Cytosolic pH measurement

Cytosolic pH (pHcyt) was measured according to Seo et al. [41]. Briefly, Me 30966 and Mel 501 cells were seeded in a 96 wells Nunc-Immuno MicroWell plate in buffered growth conditions (RPMI1640, 10% FCS). After 24 hours RPMI1640 was replaced by fresh, buffered and unbuffered medium, and cells were treated with LAN 50 μM for 24 hours. BCECF-AM was then added at a final concentration of 3 μM for 1 hour. Fluorescence intensity was measured as described for extracellular pH measurement. Standard curve was obtained by adding BCECF-AM in RPMI 1640 plus 10% FCS, buffered at different pH (5.1–7.4). pHcyt was calculated utilizing 490/440 nm fluorescence ratio values and employing the standard curve and linear equation.

In vivo *tumor growth analyses*

CB.17 SCID/SCID female mice (Harlan, Italy) were used at 4–5 weeks of age and kept under pathogen-free conditions. Mice were injected subcutaneously in the right flank with 2.5×10^5 human melanoma cells Mel 501 in 0.2 ml saline. Mice were divided in four experimental groups of five mice each. In the single drugs groups, LAN resuspended in 0.2 ml saline (DMSO 5%) was administered at a concentration of 25 mg/kg by intraperitoneal (i.p.) injection 4 days a week. PTX for injection (Taxol®) was administered i.p. at a concentration of 15 mg/kg once a week. In the combined treatment group, PTX 15 mg/kg was administered i.p. once a week after 4 days of treatment with LAN 25 mg/kg. Mice in the control group received 0.2 ml saline with DMSO 5% for 5 days a week. Tumor size (mm^3) was estimated with the formula length × $width^2$/2. At least 5 mice were used for each treatment group. In this experiment, morbidity was considered as end-point according to standard clinical criteria including oversized tumor (>1.0 cm), weight loss (>20%), rough hair coat and general illness [42].

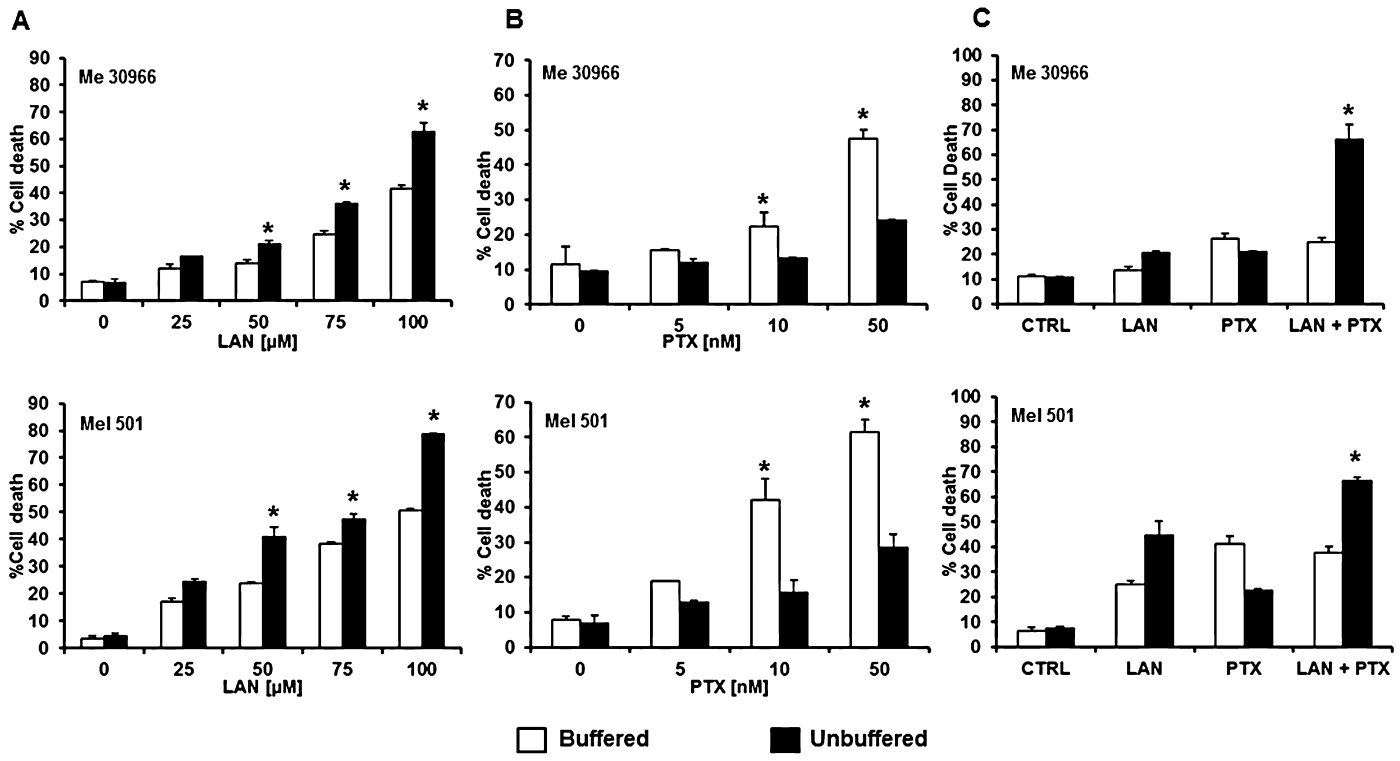

Fig. 1. Lansoprazole pretreatment enhances human melanoma cells sensitivity to Paclitaxel. LAN (A) and PTX (B) cytotoxic effects as single treatments in human melanoma cell lines *in vitro*. Me 30966 and Mel 501 cells were cultured in buffered (white columns) or unbuffered (gray columns) medium, and after 4 h of incubation different doses of the drugs were added. Cytotoxic effect was evaluated after 24 h (LAN)/48 h (PTX). (C) LAN pretreatment enhances PTX sensitivity of melanoma cell lines *in vitro*, when grown in unbuffered medium. Me 30966 and Mel 501 cells were cultured in buffered (white columns) or unbuffered (gray columns) medium, and after 4 h of incubation 50 μM LAN was added. After 24 h, the medium was replaced with fresh buffered or unbuffered medium, and 10 nM PTX was added. The cytotoxic effect was evaluated after 48 h. Columns, mean percentages of cell death of two independent experiments run in triplicate; bars indicate SD. (*) indicate $p < 0.05$.

Statistical analysis

Differences between treatment groups, both *in vitro* and *in vivo*, were analyzed by ANOVA One Way and Bonferroni t-test. Data are expressed as mean ± SD and *p* values reported are two-sided. *P* values < 0.05 were considered as statistically significant. Statistical analysis was performed with Sigmastat 2006 software.

Results

Lansoprazole pretreatment induces suboptimal dose paclitaxel sensitivity in melanoma cells

We first investigated the dose-dependent cytotoxic effects of LAN and PTX as single treatments on melanoma cells *in vitro* (Fig. 1A and B). Experiments were performed both in buffered and in unbuffered media, in order to assess the role of extracellular microenvironment acidification on the activity of the two drugs. Fig. 1A and B shows the results obtained with the two human malignant melanoma cell lines with LAN and PTX respectively. LAN induced a dose-dependent cytotoxic effect (Fig. 1A) and inhibition of cell proliferation (Supplementary Fig. S1A), always significantly higher in the unbuffered as compared to the buffered medium. Conversely, both cytotoxic (Fig. 1B) and antiproliferative (Supplementary Fig. S1B) effects obtained with PTX were significantly higher in buffered compared to unbuffered medium, at all tested doses and in all melanoma cell lines utilized. Cells were treated with PTX for 48 h because preliminary experiments had shown no effects at 24 h (not shown). This set of results suggested again that PPIs are more effective in acidic conditions, the same conditions that impair the effectiveness of the cytotoxic molecule PTX.

On the basis of previous pre-clinical experimental settings [14,15], here we performed experiments aimed at evaluating the PPI-induced sensitization of human melanoma cells to suboptimal doses of PTX. For the *in vitro* combined treatment, we used suboptimal doses of both LAN (50 μM) and PTX (10 nM). Cells were pretreated for 24 hours with LAN and then treated for an additional 48 hours with PTX. Results show that 24 h LAN pretreatment significantly increased the activity of PTX in all melanoma cell lines, exclusively when cultured in unbuffered condition (Fig. 1C). This was conceivably due to the reduced pH produced by melanoma cells when cultured in unbuffered conditions thus mimicking the spontaneous acidification of tumors. In order to assess the actual role of extracellular tumor pH in LAN and PTX sensitivity, the degree of pH changes induced in the unbuffered compared to buffered medium by melanoma cells growth was measured. After 4 hours of cell culture in unbuffered medium, the pHe, starting from 7.4, decreased of 0.46 and 0.59 points in Me 30966 and Mel 501 cells respectively. The pH of buffered medium was nearly unaffected (Supplementary Fig. S2). We know that PPIs are prodrugs needing protonation to be transformed into the active compound sulfonamide. In fact, when we co-treated in the unbuffered condition melanoma cells with suboptimal doses of both compounds, after LAN pre-treatment in unbuffered medium, the activity of PTX was 3.3 ($p < 0.05$) and 2.55 ($p < 0.05$) times in Me 30966 and Mel 501 cell lines respectively, compared to paclitaxel alone. Moreover, the combined treatment in unbuffered medium demonstrated an efficacy of 3.2 ($p < 0.01$) and 1.5 ($p < 0.05$) times higher in the two melanoma cells lines respectively, compared to LAN alone.

This result was supported by measures of extracellular, intracellular and vesicular pH before and after PPI treatment, showing that in unbuffered medium LAN was able to significantly acidify the cytosol and alkalinize extracellular microenvironment and internal vesicles of the treated cells (Table 1). The results of the recovery

Table 1
Extracellular, cytosolic and vesicular pH.

Cell line	Treatment	Buffered medium			Unbuffered medium		
		Extracellular pH	Cytosolic pH	Vesicular pH	Extracellular pH	Cytosolic pH	Vesicular pH
Me 30966	Saline	7.08 ± 0.046	7.52 ± 0.16	4.89 ± 0.06	6.77 ± 0.057	7.20 ± 0.16	5.41 ± 0.07
	Lansoprazole	7.28 ± 0.021	7.20 ± 0.14	5.01 ± 0.10	7.04 ± 0.084	6.38 ± 0.13	5.74 ± 0.07
Mel 501	Saline	7.09 ± 0.039	7.38 ± 0.15	5.00 ± 0.05	6.84 ± 0.022	7.41 ± 0.14	5.28 ± 0.08
	Lansoprazole	7.22 ± 0.040	7.47 ± 0.20	5.16 ± 0.06	7.07 ± 0.038	6.87 ± 0.13	5.82 ± 0.09

Extracellular, vesicular and cytosolica pH values of Me 30966 and Mel 501 cells treated with Lansoprazole in comparison with untreated cells in unbuffered and buffered growth media. Saline: untreated cells; Lansoprazole: lansoprazole 50 μM for 24 h. Values indicate mean values of three different experiments ± standard deviation (SD).

experiments (Table 2) showed that cells, cultured in unbuffered RMPI and treated with 24 h LAN 50 μM, had reduced ability to acidify the medium following LAN treatment, thus increasing the PTX effectiveness. Moreover, treatment with LAN not only prevented acidification of the extracellular medium, but also produced a normalization toward physiological levels of pH gradient between the cytosol and the extracellular environment, and between the cytosol and internal vesicles (Table 1).

Lansoprazole pretreatment enhances paclitaxel sensitivity in SCID mice engrafted with human tumor cells

To assess the potential clinical relevance of the *in vitro* results, we performed *in vivo* experiments in a human/mouse model system represented by CB.17 SCID/SCID mice injected subcutaneously with human melanoma cells (Mel501). This model was chosen as particularly suited for evaluating direct effects of drugs on human tumors, without possible interference from host cell components. Four days after injection of melanoma cells, when tumors were present as palpable/visible lesion, the different treatments were administered. In order to evaluate potential side effects of PTX and LAN, treated mice were monitored for the duration of the experiment for body weight, hair ruffling and presence of diarrhea, and no signs of toxicity were detected. The schedules of treatment were repeated for ten weeks. Subsequently, while treatment continued in the single drug groups, in the group of combined treatment the administration of PTX was interrupted, and only LAN 4 days per week continued as maintenance therapy. Tumor growth was monitored 2 times per week for 100 days (Fig. 2A); by that time, all animals in the control group had to be euthanized. Survival rates were monitored until week 18 (Fig. 3D). In line with *in vitro* data, PTX following LAN pretreatment significantly reduced melanoma *in vivo* growth compared to the control group, and also to the single treatment groups (Fig. 2A). Fig. 2B shows tumors explanted at the end of the experiment, representative for each treatment group. Moreover, we have observed that, following the interruption of the administration of PTX in the combined treatment group, tumors size remained significantly lower with respect to the other treatment groups until the end of the experiment (Fig. 3A and B): this result suggested that LAN might be used in maintenance therapy of melanoma following LAN/PTX combined treatment. The inhibition of tumor growth observed in human melanoma xenografts when treated with PTX following pretreatment with LAN was consistent with a lower body weight loss over treatment period (Fig. 3C) and a significant increase of animals survival rate (Fig. 3D). Histochemical analysis of Mel501 melanoma lesions revealed the presence of large necrotic areas in the combined treatment group, compared to untreated animals (not shown), suggesting that the effect of the combined therapy was consistent with the cytotoxic effect shown in the *in vitro* experiments (Fig. 1C).

Table 2
Extracellular pH (recovery).

Cell Line	Treatment	Bufferd medium	Unbufferd medium
		Extracellular pH	Extracellular pH
Me 30966	Saline	7.29 ± 0.20	6.71 ± 0.056
	Lansoprazole	7.22 ± 0.30	7.22 ± 0.028
Mel 501	Saline	7.35 ± 0.15	6.75 ± 0.155
	Lansoprazole	7.33 ± 0.078	7.20 ± 0.049

Extracellular pH values of Me 30966 and Mel 501 cells treated with Lansoprazole in comparison with untreated cells in buffered and unbuffered growth media during recovery test. Saline: untreated cells; Lansoprazole: lansoprazole 50 μM for 24 h. Values indicate mean values of three different experiments ± standard deviation (SD).

Discussion

Metastatic melanoma is usually scarcely or not responsive to treatment with cytotoxic chemotherapeutic drugs such as alkylating agents (dacarbazine), platinum analogues (cisplatin, carboplatin) and antimicrotubular agents (PXT) [5]. Moreover, these drugs are associated with high toxicity, especially when administered in combinations [43]. Thus, alternative treatment options for melanoma are strongly needed. While almost neglected, a very effective mechanism of tumor resistance to therapy is micro-environmental acidity, that hampers through protonation the cytotoxic drug entry within tumor cells [7,44]. We previously showed that pre-treatment with a class of PPIs induced sensitivity to chemotherapeutics in a variety of human cancer cells and cancers, including melanoma [14]. However, we also showed that PPIs had a clear anti-tumor effect when used at high dosages [16,17]. The aim of the present study was to determine whether the well tolerated PPI LAN might be able to potentiate the cytotoxicity of suboptimal doses of the taxane PTX against human metastatic melanoma cells, in order to achieve a more effective and less toxic treatment strategy. PTX is one of the first-line agents that are effective for the treatment of a wide range of cancers, including lung, ovarian, breast, prostate, head and neck cancer, and advanced forms of Kaposi's sarcoma. In metastatic melanoma PTX is used as a second-line therapy, both as monotherapy [45] or as part of a combination with other therapies [46]. In this work we show that PTX and the PPI LAN, used in combination at suboptimal doses, have a superior, higher than addictive, efficacy against metastatic melanoma cells, as compared to the single treatments, both *in vitro* (Fig. 1C) and *in vivo* (Figs. 2 and 3). We also show that acidity plays an important role on the antitumor activity of these substances, used either alone or in combination, but with opposite effect inducing an increase of the PPI action and a decrease in PTX effect. Indeed, melanoma tumor cells, grown in unbuffered medium, create an acidic extracellular environment (Supplementary Fig. S2A and B) which, while detrimental for PTX activity, is necessary for the synergistic effect of PTX following pretreatment with LAN, presumably because it is involved in the activation of this weak base prodrug. These data are in agreement with both a study demonstrating in breast carcinoma cell line MCF-7 that a low extracellular pH induces higher resistance to PTX [47], and our previous results showing that antitumor PPIs effects are pH-dependent [17]. Unbuffered medium has been used in *in vitro* experiments as a model of spontaneous acidification of the tumor

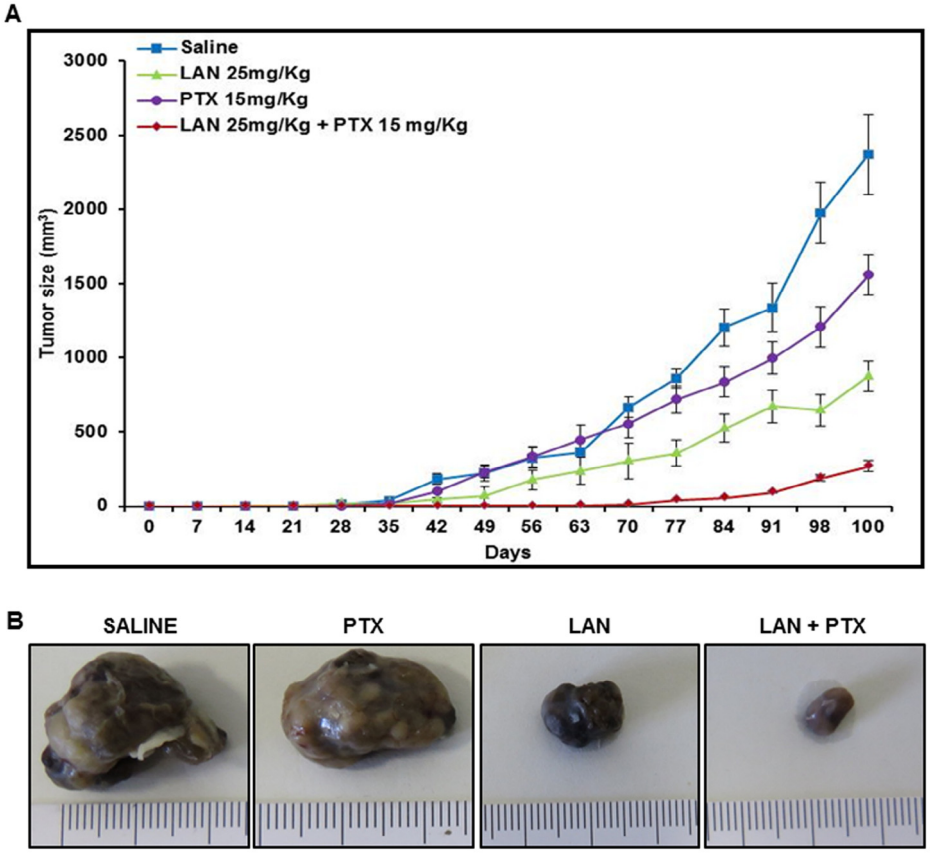

Fig. 2. Lansoprazole pretreatment enhances the *in vivo* effects of Paclitaxel in SCID mice xenografted with human melanoma cells. LAN (25 mg/kg) pretreatment enhances PTX (15 mg/kg) sensitivity in SCID mice engrafted with human melanoma cells. Mice engrafted with human melanoma cells Mel 501, at the time of tumor appearance were subdivided in four groups: the control group was left untreated; the single drug groups received one 25 mg/kg LAN for 4 days per week, and the other 15 mg/kg PTX for 1 day per week; finally the combined treatment group received 15 mg/kg PTX for 1 day after 4 days of 25 mg/kg LAN per week. (A) Tumor growth was monitored 2 times per week for 100 days; after 10 weeks PTX was discontinued in the combined treatment group and LAN alone was continued as maintenance therapy; bars indicate SE. (B) Tumors explanted at the end of the experiment, representative for each treatment group.

micro-environment. Previous investigation in a human melanoma/SCID mouse xenograft model has shown that treatment with PPI led to an increase in the extracellular pH and a reduction in the cytosolic pH, in turn leading to a significant reduction of the tumor pH gradients that returned to the original values following the stop of the *in vivo* PPI treatment [17]. This last is the most suitable condition for the entry of a weak base drug within a cell. In another set of experiments performed in a syngenic mouse melanoma model we have again shown that PPI *in vivo* treatment induced a significant increase in the extracellular pH thus increasing the effectiveness of both an adoptive immunotherapy and the immune reaction against the melanoma [48].

This study also shows that LAN treatment induces a decrease in cytosolic pH and an increase in extracellular and vesicular pH exclusively in unbuffered conditions (Table 1). Furthermore, as shown in the recovery experiments (Table 2), cells treated for 24 hours with LAN were shown to lose the ability to acidify the medium, thus contributing to the increased PTX performance.

Importantly, we have shown here that such an approach of utilizing the well tolerated PPI LAN followed by a suboptimal (and consequently less toxic) dose of PTX allows *in vivo* to obtain very encouraging results. Indeed, using PTX at a dose of 15 mg/kg, which is well tolerated in mice [49-51], we observe a striking difference among the results obtained with PTX alone or combined with LAN pretreatment, in terms of tumor size, weight loss over treatment period, and percentage of surviving animals (Figs. 2 and 3). Interestingly, after PTX was discontinued in the combined treatment group, tumors size remained significantly lower with respect to the other groups until the end of the experiment, suggesting that single LAN treatment can be well exploited as maintenance therapy for melanoma patients, following combined treatment (Fig. 3A and B).

In conclusion, tumor acidosis is increasingly considered an important determinant of tumor progression and drug resistance, and our result further strengthens the potential of including PPIs in combination chemotherapy as an acidity targeted, anti-acid approach, particularly in melanoma patients with recurrent and/or metastatic disease, that are presently without a real line of therapy. Indeed, PPIs are a class of potent anti-acidic drugs, designed for the treatment of peptic diseases, that have been used by billions of people worldwide in the past decades, without significant side effects, even at high dosages (as in patients with Zollinger–Hellison syndrome). PPIs are being also extensively investigated for their potential to reduce tumor acidity and overcome acid-related chemoresistance both *in vitro* and *in vivo* [14,15,19–21]. Our results demonstrate that suboptimal doses of PTX in combination with LAN can be effective against tumor cells and tumors deriving from human metastatic melanoma, which is usually poorly responsive to PTX. The results we obtained in human melanoma can also be important for different tumor histotypes which are more sensitive to PTX, with the purpose to increase the efficacy, but also to reduce or avoid systemic toxicity.

Acknowledgement

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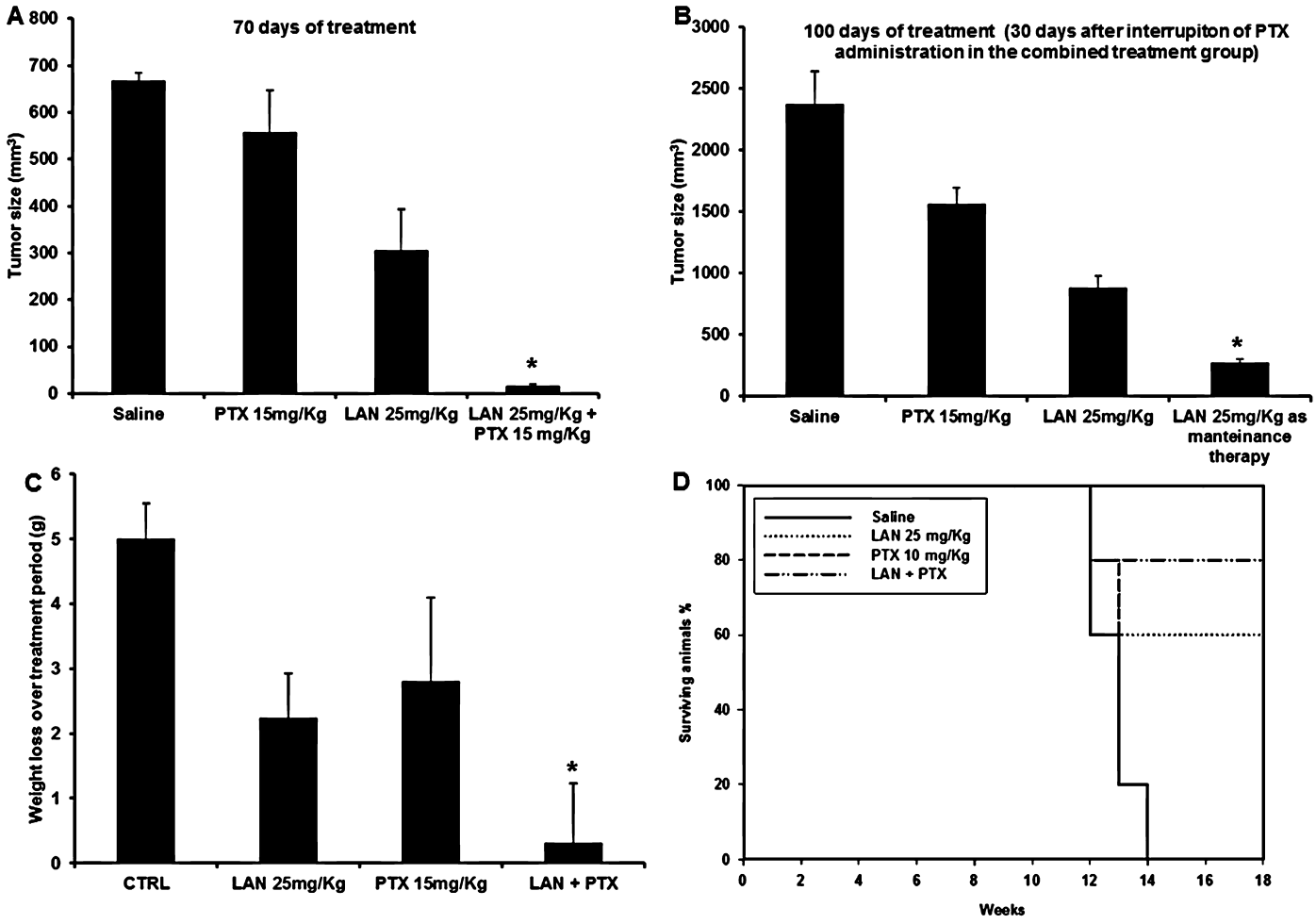

Fig. 3. *In vivo* effects of Lansoprazole treatment in combination with Paclitaxel. (A) Tumors size is shown for the 4 groups of mice after 10 weeks of treatment (until PTX was discontinued in the combined treatment group). (B) Tumors size is shown for the 4 groups of mice at the end of the observation period (100 days). (C) The absolute weight loss (weight at the beginning of treatments – weight at day 100) is shown for the 4 groups of mice. Bars indicate SD. (*) indicate $p < 0.05$. (D) Survival rates of the 4 groups of human melanoma-engrafted SCID mice.

Conflict of interest

None.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2014.10.017.

References

[1] A. Jemal, R. Siegel, J. Xu, E. Ward, Cancer statistics, 2010, CA Cancer J. Clin. 60 (2010) 277–300.
[2] B. Bandarchi, L. Ma, R. Navab, A. Seth, G. Rasty, From melanocyte to metastatic malignant melanoma, Dermatol. Res. Pract. 2010 (2010) doi:10.1155/2010/583748 [Epub 2010 Aug 11].
[3] B.N. Bristow, J. Casil, F. Sorvillo, R. Basurto-Davila, T. Kuo, Melanoma-related mortality and productivity losses in the USA, 1990–2008, Melanoma Res. 23 (2013) 331–335.
[4] S. Bhatia, S.S. Tykodi, J.A. Thompson, Treatment of metastatic melanoma: an overview, Oncology (Williston Park) 23 (2009) 488–496.
[5] K.G. Chen, J.C. Valencia, J.P. Gillet, V.J. Hearing, M.M. Gottesman, Involvement of ABC transporters in melanogenesis and the development of multidrug resistance of melanoma, Pigment Cell. Melanoma Res. 22 (2009) 740–749.
[6] K. Rass, W. Tilgen, Treatment of melanoma and nonmelanoma skin cancer, Adv. Exp. Med. Biol. 624 (2008) 296–318.
[7] A. De Milito, S. Fais, Tumor acidity, chemoresistance and proton pump inhibitors, Future Oncol. 1 (2005) 779–786.
[8] R.A. Cairns, I.S. Harris, T.W. Mak, Regulation of cancer cell metabolism, Nat. Rev. Cancer 11 (2011) 85–95.
[9] R.A. Gatenby, R.J. Gillies, Why do cancers have high aerobic glycolysis?, Nat. Rev. Cancer 4 (2004) 891–899.
[10] S. Harguindey, J.L. Arranz, M.L. Wahl, G. Orive, S.J. Reshkin, Proton transport inhibitors as potentially selective anticancer drugs, Anticancer Res. 29 (2009) 2127–2136.
[11] S. Fais, Proton pump inhibitor-induced tumour cell death by inhibition of a detoxification mechanism, J. Intern. Med. 267 (2010) 515–525.
[12] A. De Milito, M.L. Marino, S. Fais, A rationale for the use of proton pump inhibitors as antineoplastic agents, Curr. Pharm. Des. 18 (2012) 1395–1406.
[13] L. Olbe, E. Carlsson, P. Lindberg, A proton-pump inhibitor expedition: the case histories of omeprazole and esomeprazole, Nat. Rev. Drug Discov. 2 (2003) 132–139.
[14] F. Luciani, M. Spada, A. De Milito, et al., Effect of proton pump inhibitor pretreatment on resistance of solid tumors to cytotoxic drugs, J. Natl. Cancer Inst. 96 (2004) 1702–1713.
[15] S. Ferrari, F. Perut, F. Fagioli, et al., Proton pump inhibitor chemosensitization in human osteosarcoma: from the bench to the patients' bed, J. Transl. Med. 11 (2013) 268.
[16] A. De Milito, E. Iessi, M. Logozzi, et al., Proton pump inhibitors induce apoptosis of human B-cell tumors through a caspase-independent mechanism involving reactive oxygen species, Cancer Res. 67 (2007) 5408–5417.
[17] A. De Milito, R. Canese, M.L. Marino, et al., pH-dependent antitumor activity of proton pump inhibitors against human melanoma is mediated by inhibition of tumor acidity, Int. J. Cancer 127 (2010) 207–219.
[18] M.L. Marino, S. Fais, M. Djavaheri-Mergny, et al., Proton pump inhibition induces autophagy as a survival mechanism following oxidative stress in human melanoma cells, Cell. Death Dis. 1 (2010) e87.

[19] A. Udelnow, A. Kreyes, S. Ellinger, K. Landfester, P. Walther, T. Klapperstueck, et al., Omeprazole inhibits proliferation and modulates autophagy in pancreatic cancer cells, PLoS ONE 6 (2011) e20143.
[20] M. Chen, X. Zou, H. Luo, J. Cao, X. Zhang, B. Zhang, et al., Effects and mechanisms of proton pump inhibitors as a novel chemosensitizer on human gastric adenocarcinoma (SGC7901) cells, Cell Biol. Int. 33 (2009) 1008–1019.
[21] M. Chen, S.L. Huang, X.Q. Zhang, B. Zhang, H. Zhu, V.W. Yang, et al., Reversal effects of pantoprazole on multidrug resistance in human gastric adenocarcinoma cells by down-regulating the V-ATPases/mTOR/HIF-1alpha/P-gp and MRP1 signaling pathway in vitro and in vivo, J. Cell. Biochem. 113 (2012) 2474–2487.
[22] W. Shen, X. Zou, M. Chen, Y. Shen, S. Huang, H. Guo, et al., Effect of pantoprazole on human gastric adenocarcinoma SGC7901 cells through regulation of phosphoLRP6 expression in wnt/beta-catenin signaling, Oncol. Rep. 30 (2013) 851–855.
[23] M. Yeo, D.K. Kim, Y.B. Kim, T.Y. Oh, J.E. Lee, S.W. Cho, et al., Selective induction of apoptosis with proton pump inhibitor in gastric cancer cells, Clin. Cancer Res. 10 (2004) 8687–8696.
[24] S. Avnet, G. Di Pompo, S. Lemma, M. Salerno, F. Perut, G. Bonuccelli, et al., V-ATPase is a candidate therapeutic target for Ewing sarcoma, Biochim. Biophys. Acta 2013 (1832) 1105–1116.
[25] F. Perut, S. Avnet, C. Fotia, S.R. Baglio, M. Salerno, S. Hosogi, et al., V-ATPase as an effective therapeutic target for sarcomas, Exp. Cell Res. 320 (2014) 21–32.
[26] S. Zhang, Y. Wang, S.J. Li, Lansoprazole induces apoptosis of breast cancer cells through inhibition of intracellular proton extrusion, Biochem. Biophys. Res. Commun. 448 (2014) 424–429.
[27] E.P. Spugnini, A. Baldi, S. Buglioni, F. Carocci, G.M. de Bazzichini, G. Betti, et al., Lansoprazole as a rescue agent in chemoresistant tumors: a phase I/II study in companion animals with spontaneously occurring tumors, J. Transl. Med. 9 (2011) 221.
[28] E.P. Spugnini, S. Buglioni, F. Carocci, M. Francesco, B. Vincenzi, M. Fanciulli, et al., High dose lansoprazole combined with metronomic chemotherapy: a phase I/II study in companion animals with spontaneously occurring tumors, J. Transl. Med. 21 (2014) 225.
[29] C. Federici, F. Petrucci, S. Caimi, et al., Exosome release and low pH belong to a framework of resistance of human melanoma cells to cisplatin, PLoS ONE 9 (2014) e88193.
[30] M.C. Wani, H.L. Taylor, M.E. Wall, P. Coggon, A.T. McPhail, Plant antitumor agents. VI. the isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia, J. Am. Chem. Soc. 93 (1971) 2325–2327.
[31] M.A. Jordan, L. Wilson, Microtubules as a target for anticancer drugs, Nat. Rev. Cancer 4 (2004) 253–265.
[32] S.S. Legha, S. Ring, N. Papadopoulos, M. Raber, R.S. Benjamin, A phase II trial of taxol in metastatic melanoma, Cancer 65 (1990) 2478–2481.
[33] M. Kavallaris, J.P. Annereau, J.M. Barret, Potential mechanisms of resistance to microtubule inhibitors, Semin. Oncol. 35 (2008) S22–S27.
[34] I.A. Cree, M.H. Neale, N.E. Myatt, et al., Heterogeneity of chemosensitivity of metastatic cutaneous melanoma, Anticancer Drugs 10 (1999) 437–444.
[35] I.F. Kretzer, D.A. Maria, R.C. Maranhao, Drug-targeting in combined cancer chemotherapy: tumor growth inhibition in mice by association of paclitaxel and etoposide with a cholesterol-rich nanoemulsion, Cell. Oncol. (Dordr) 35 (2012) 451–460.
[36] C. Zimpfer-Rechner, U. Hofmann, R. Figl, J.C. Becker, U. Trefzer, I. Keller, et al., Randomized phase II study of weekly paclitaxel versus paclitaxel and carboplatin as second-line therapy in disseminated melanoma: a multicentre trial of the dermatologic co-operative oncology group (DeCOG), Melanoma Res. 13 (2003) 531–536.
[37] V. Vukovic, I.F. Tannock, Influence of low pH on cytotoxicity of paclitaxel, mitoxantrone and topotecan, Br. J. Cancer 75 (1997) 1167–1172.
[38] S.J. Reshkin, A. Bellizzi, R.A. Cardone, M. Tommasino, V. Casavola, A. Paradiso, Paclitaxel induces apoptosis via protein kinase A- and p38 mitogen-activated protein-dependent inhibition of the Na^+/H^+ exchanger (NHE) NHE isoform 1 in human breast cancer cells, Clin. Cancer Res. 9 (2003) 2366–2373.
[39] L. Xiaodong, et al., The growth and metastasis of human hepatocellular carcinoma xenografts are inhibited by small interfering RNA targeting to the subunit ATP6L of proton pump, Cancer Res. 65 (2005) 6843–6849.
[40] S. Ohkuma, B. Poole, Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents, Proc. Natl. Acad. Sci. U.S.A. 75 (1978) 3327–3331.
[41] J.T. Seo, M.C. Steward, J.B. Larcombe-McDouall, L.J. Cook, R.M. Case, Continuous fluorometric measurement of intracellular pH and Ca^{2+} in perfused salivary gland and pancreas, Pflugers Arch. 426 (1994) 75–82.
[42] D.B. Morton, P.H. Griffiths, Guidelines on the recognition of pain, distress and discomfort in experimental animals and a hypothesis for assessment, Vet. Rec. 116 (1985) 431–436.
[43] R. Mouawad, M. Sebert, J. Michels, J. Bloch, J.P. Spano, D. Khayat, Treatment for metastatic malignant melanoma: old drugs and new strategies, Crit. Rev. Oncol. Hematol 74 (2010) 27–39.
[44] S. Fais, A. De Milito, H. You, W. Qin, Targeting vacuolar H^+-ATPases as a new strategy against cancer, Cancer Res. 67 (2007) 10627–10630.
[45] A.Y. Bedikian, C. Plager, N. Papadopoulos, O. Eton, J. Ellerhorst, T. Smith, Phase II evaluation of paclitaxel by short intravenous infusion in metastatic melanoma, Melanoma Res. 14 (2004) 63–66.
[46] A. Hauschild, S.S. Agarwala, U. Trefzer, et al., Results of a phase III, randomized, placebo-controlled study of sorafenib in combination with carboplatin and paclitaxel as second-line treatment in patients with unresectable stage III or stage IV melanoma, J. Clin. Oncol. 27 (2009) 2823–2830.
[47] D. Tavares-Valente, F. Baltazar, R. Moreira, O. Queiros, Cancer cell bioenergetics and pH regulation influence breast cancer cell resistance to paclitaxel and doxorubicin, J. Bioenerg. Biomembr. 45 (2013) 467–475.
[48] A. Calcinotto, P. Filippazzi, M. Grigioni, M. Iero, A. De Milito, A. Ricupito, et al., Modulation of microenvironment acidity reverses anergy in human and murine tumor-infiltrating T lymphocytes, Cancer Res. 72 (2012) 2746–2756.
[49] J. Lu, Y. Huang, W. Zhao, R.T. Marquez, X. Meng, J. Li, et al., PEG-derivatized embelin as a nanomicellar carrier for delivery of paclitaxel to breast and prostate cancers, Biomaterials 34 (2013) 1591–1600.
[50] X. Zhang, H.M. Burt, G. Mangold, D. Dexter, D. Von Hoff, L. Mayer, et al., Anti-tumor efficacy and biodistribution of intravenous polymeric micellar paclitaxel, Anticancer Drugs 8 (1997) 696–701.
[51] A. Sharma, R.M. Straubinger, Novel taxol formulations: preparation and characterization of taxol-containing liposomes, Pharm. Res. 11 (1994) 889–896.