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pH-dependent antitumor activity of proton pump inhibitors against human melanoma is mediated by inhibition of tumor acidity

Angelo De Milito[1], Rossella Canese[2], Maria Lucia Marino[1], Martina Borghi[1], Manuela Iero[3], Antonello Villa[4], Giulietta Venturi[1], Francesco Lozupone[1], Elisabetta Iessi[1], Mariantonia Logozzi[1], Pamela Della Mina[3,4], Mario Santinami[5], Monica Rodolfo[3], Franca Podo[2], Licia Rivoltini[3] and Stefano Fais[1]

[1] Department of Therapeutic Research and Medicines Evaluation, Unit of Antitumor Drugs, Istituto Superiore di Sanità, Rome, Italy
[2] Department of Cell Biology and Neurosciences, Unit of Molecular and Cellular Imaging, Istituto Superiore di Sanità, Rome, Italy
[3] Unit of Immunotherapy of Human Tumors, Fondazione IRCCS Istituto Nazionale Tumori, Milan, Italy
[4] Microscopy and Image Analysis Consortium, University of Milano-Bicocca, Monza, Italy
[5] Unit of Melanoma and Sarcoma, Fondazione IRCCS Istituto Nazionale Tumori, Milan, Italy

Metastatic melanoma is associated with poor prognosis and still limited therapeutic options. An innovative treatment approach for this disease is represented by targeting acidosis, a feature characterizing tumor microenvironment and playing an important role in cancer malignancy. Proton pump inhibitors (PPI), such as esomeprazole (ESOM) are prodrugs functionally activated by acidic environment, fostering pH neutralization by inhibiting proton extrusion. We used human melanoma cell lines and xeno-transplated SCID mice to provide preclinical evidence of ESOM antineoplastic activity. Human melanoma cell lines, characterized by different mutation and signaling profiles, were treated with ESOM in different pH conditions and evaluated for proliferation, viability and cell death. SCID mice engrafted with human melanoma were used to study ESOM administration effects on tumor growth and tumor pH by magnetic resonance spectroscopy (MRS). ESOM inhibited proliferation of melanoma cells *in vitro* and induced a cytotoxicity strongly boosted by low pH culture conditions. ESOM-induced tumor cell death occurred *via* rapid intracellular acidification and activation of several caspases. Inhibition of caspases activity by pan-caspase inhibitor z-vad-fmk completely abrogated the ESOM-induced cell death. ESOM administration (2.5 mg kg^{-1}) to SCID mice engrafted with human melanoma reduced tumor growth, consistent with decrease of proliferating cells and clear reduction of pH gradients in tumor tissue. Moreover, systemic ESOM administration dramatically increased survival of human melanoma-bearing animals, in absence of any relevant toxicity. These data show preclinical evidence supporting the use of PPI as novel therapeutic strategy for melanoma, providing the proof of concept that PPI target human melanoma modifying tumor pH gradients.

Despite the major efforts made to identify novel therapeutic tools for metastatic melanoma, durable regressions are still rare events in patients with advanced disease, and no significant benefit in survival has been so far achieved.[1] The identification of alternative strategies based on different rationale to control disease progression remains mandatory in the field of melanoma research.

It is becoming increasingly evident that the chronic destruction of cellular homeostasis occurring in cancer cells by metabolic alterations including glycolysis and intracellular

Key words: proton pump inhibitors, tumor acidity, magnetic resonance spectroscopy, human melanoma, SCID mice, vacuolar ATPase

Abbreviations: 3-APP: 3-aminopropyl phosphonate; BCECF-AM: 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester; ESOM: esomeprazole; MRS: magnetic resonance spectroscopy; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PARP-1: poly (ADP-ribose) polymerase; pHe: extracellular pH; pHi: intracellular pH; PPI: proton pump inhibitors; SRB: sulforhodamine B; V-ATPase: vacuolar ATPase

Additional Supporting Information may be found in the online version of this article.

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Correspondence to: Angelo De Milito, PhD, Department of Therapeutic Research and Medicines Evaluation, Unit of Antitumor Drugs, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Rome, Italy, Tel.: +39-06-49902153; Fax: +39-06-49902436-3691, E-mail: angelo.demilito@iss.it

alkalinisation associated with microenvironment acidification, is a main factor driving tumor progression, invasion and metastases.[2–5] This novel concept is promoting a more integrated view of cancer therapy aimed at targeting and/or exploiting features of tumor microenvironment to kill cancer cells. Acidity of human solid tumors is involved in tumor progression and malignancy,[5–7] mostly by inducing a selection of cells adapted through an altered metabolism to survive in hostile conditions.[2] Recent *in vivo* studies on animal models and also in patients with oral cancer have shown an acidic extracellular pH (pHe) and neutral-to-alkaline intracellular pH (pHi) in tumor lesions.[8–10] The acidic tumor pHe drives proliferation, favors chemoresistance and promotes metastatic potential[11–15] whereas maintenance of alkaline pHi sustains resistance to cytotoxicity.[16,17]

The abnormal pH gradient characterizing tumor cells is finely tuned by different ion/proton pumps including the vacuolar ATPase (V-ATPase),[3,18] whose expression and activity are enhanced in human tumors.[19,20] Gene knock-down of the V-ATPase subunit c reduces growth of hepatocellular carcinoma and sensitizes breast cancer cells to chemotherapeutics, providing synergistic effects on tumor growth.[21,22] Hence, pharmacological targeting of V-ATPase to regulate tumor pH or overcome chemoresistance has been recently proposed as a novel anticancer strategy.[3,4,18,23]

Inhibition of V-ATPase activity can also be achieved by treatment with proton pump inhibitors (PPI)[24,25] and we have recently shown that treatment with PPI sensitizes drug-resistant human tumors to chemotherapeutics and inhibits growth of human B cell tumors.[14,26] Moreover, PPI were shown to selectively induce apoptosis in gastric cancer cells.[27] PPI, originally identified as specific inhibitors of gastric acid pump are pro-drugs which need acidic pH to be transformed in the active molecule (tetracyclic sulfenamide), thus they may accumulate in acidic tissues and act locally. PPI have been used for decades as pivotal treatment of peptic diseases, with minimal side effects[28] even when administered at high dose and by chronic schedules as required in patients with Zollinger-Ellison syndrome.[29,30] The safety profiles of PPI and our preclinical study[14] represented the rationale for 2 clinical trials presently ongoing at the National Tumor Institute (Milan) and the Istituto Ortopedico Rizzoli (Bologna) in Italy, with the endpoint of evaluating the chemo-sensitizing effect of PPI in melanoma and osteosarcoma patients.

A rationale for the anti-melanoma effect of PPI is that they may inhibit a homeostatic mechanism helping cancer cell survival by disposing of H+ and acidic metabolites.[19,31–33] Moreover, PPI induce cell death in gastric cancer cells and B cell tumors.[26,27] Interestingly, specific targeting of solid tumor lesions by systemic PPI administration should be warranted by acidic pH of cancer lesions,[34] possibly acting as a preferential site of PPI activation for local exertion of anti-tumor activity.

Here, we extensively investigated in a preclinical setting the activity of esomeprazole (ESOM), a prototype of PPI, as antineoplastic agent for human melanoma. Data reported here clearly show that treatment with ESOM may represent a promising and safe therapeutic strategy against melanoma.

Material and Methods

Drugs

Omeprazole and esomeprazole sodium salts (AstraZeneca, Sweden) were resuspended 2 mg ml^{-1} in saline before use. RPMI 1640, antibiotics and fetal calf serum (FCS) were from Cambrex (Milano, Italy).

Cell culture

Human melanoma cell lines were previously established from melanoma lesions of patients (Istituto Nazionale dei Tumori) and characterized,[35] melanoma cell lines Mel501, WM902 and WM793 (were a gift from Dr. Meenhard Herlynt, Wistar Institute, PA). Cell lines were cultured using RPMI 1640 in presence of 10% FCS and antibiotics. Experiments were performed in buffered medium (pH 7.4), unbuffered medium (w/o sodium bicarbonate, initial pH 7.2) or buffered acidic medium (pH 5.0 or 6.0) as specified through the text.

Cell proliferation/viability

Melanoma cells were plated in 96-well plates in buffered, unbuffered or acidic medium (pH 6.0) and the day after ESOM was added. After 24 hr thymidine incorporation was measured by adding 1μCi/well [^{3}H]-thymidine (Pierce Biotechnology, Rockford, IL). ESOM effect on cell viability was determined in cells cultured in unbuffered medium 24 hr after PPI treatment using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Milano, Italy). The sulforhodamine B (SRB) has also been used to evaluate ESOM cytotoxicity in some melanoma cell lines (Sigma-Aldrich, Milano, Italy).

Cell death and intracellular pH determination

Cells were seeded in unbuffered or acidic pH medium in 12 well-plates. The next day ESOM was added and 24 hr later cells were collected for analysis. At time of ESOM treatment, the pH of unbuffered medium ranged 6.6–6.8 depending on the cell lines while the pH of acidic medium remained unchanged. The involvement of specific caspases in ESOM-mediated cell death was evaluated by using the specific caspase inhibitors Z-VDVAD-fmk (caspase-2), Z-DEVD-fmk (caspase-3), Z-IETD-fmk (caspase-8) and Z-LEHD-fmk (caspase-9) diluted 1:200 (Alexis Biochemicals, Firenze, Italy). The pan-caspases inhibitor Z-VAD-fmk was used 10 μM and all caspases inhibitors were incubated with the cells 2 hr prior the addition of ESOM. Pepstatin A (Roche Molecular Biochemicals, Milano), CA-074 (Calbiochem, Milano) and E64d (Sigma Aldrich) were respectively used 100, 10, and 25 μM. Cell death was determined by Annexin-V-FITC/propidium iodide staining (Alexis). Cells were sorted on a Becton

Dickinson FACScan and analyzed with CellQuestPro (Becton Dickinson Systems).

Intracellular pH was evaluated by flow cytometry using the pH-sensitive fluorescent probe BCECF-AM (Molecular Probes) as described in details previously.[26]

Caspase activity

Caspases activity was measured using the caspases-specific CaspGLOW-FITC kit (MBL International, Japan). Briefly, 0.1 $\times$ 10^6 cells untreated or treated with ESOM were collected and washed once in PBS. The cells were then resuspended into 100 μl Wash Buffer containing 0.33 μl of the caspase-specific FITC-conjugated peptide and incubated at 37°C for 60 min. After 1 wash in Wash Buffer the cells were analyzed by FACS evaluating the percentage of FITC-positive cells.

Flow cytometry

Analysis of V-ATPase subunits A and a expression was performed on cells fixed with 2% paraformaldehyde and permeabilised with 0.05% Triton-X. Following incubation with subunit-specific antibodies (Molecular Probes) and anti-mouse antibody conjugated with AlexaFluor-488, cells were analyzed by FACS using CellQuestPro software and V-ATPase expression was quantified by mean fluorescence intensity.

Western blot

Me30966 cells (both adherent and floating cells) were collected and lysed in RIPA buffer (150 mM sodium chloride 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0). For Western blotting, 30 μg total cell lysates were separated on 12% Bis-Tris precasted gels (Invitrogen) and transferred to PVDF membrane (Amersham). Membranes were probed with primary antibodies to active caspase-3 and PARP-1 (BD Pharmingen) and beta-actin (Cell Signaling). After probing with HRP-conjugated anti-mouse or anti-rabbit antibodies (Cell Signaling) specific staining was visualized by enhanced chemiluminescence (ECL) (Pierce, Rockford, IL).

Mutational profile and protein expression

A description of the protein expression analysis as wells as the PCR and sequencing analyses for determining the mutational profiles of the cell lines used has been previously reported in details.[35]

Animal experiments

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 5×10^6 human melanoma cells in 0.2 ml saline. ESOM was orally administered by gavage and at least 8 mice were used for each treatment group. Tumor size (mm^3) was estimated with the formula length $\times$ width^2 /2. In 1 experiment with Mel501 cells, morbidity was considered as end-point according to standard clinical criteria including oversized tumor (>1 cm), weight loss (>20%), rough hair coat and general illness.[36] For histological analysis, tumors were fixed in formalin and embedded in paraffin. Four-micrometer sections stained with haematoxylin/eosin and analyzed for Ki-67 expression. Animal care was conformed to European Council Directive 86/609/EEC and the study was approved by institutional review board.

In vivo magnetic resonance spectroscopy (MRS)

Tumor pHe value was measured from chemical shift difference between the exogenous cell impermeant ^{31}P reporter 3-aminopropyl phosphonate (3-APP) resonance and that of α-ATP at -7.57 ppm. The pHi was measured from chemical shift difference between inorganic phosphate (Pi) and α-ATP. Human melanoma cells (Me15392) were injected s.c. in the dorsum of SCID mice and once tumors became larger than 600 μl, ESOM (2.5 mg kg^{-1}) was administered by gavage. The 3-APP probe (128 mg kg^{-1}) was administered i.p. immediately prior to MRI/MRS analyses.

A Varian Inova 200/183 MRI/MRS system for small animals operating at 4.7 T was used for MRI/MRS analysis. A 1 cm 2-turn or a 2 cm 3-turn ^{31}P surface coil was used in combination with a volume (6 cm diameter) ^{1}H coil. T1-weighted gradient-echo multislice contiguous images (TR/TE = 123/4.3 msec, $\alpha = 20°$, thickness = 2 mm, 8 averages, FOV = 3 $\times$ 3 cm^2) were acquired to localize the tumor. ^{1}H localized spectra were used to increase the signal resolution within the tumor (^{1}H PRESS, TR/TE = 2,000/23 msec). ^{31}P localized spectra were acquired from the tumor with ISIS (TR/TM = 2,000/80 msec, 2,048 averages, VOI ranging from 300 to 400 μl) or ^{1}H-decoupled pulse-acquire sequence (TR = 3,000 ms, $\alpha = 25°$, garp, 256 averages).

Statistical analysis

Differences between groups were analyzed by Mann-Whitney test, student T test or by ANOVA as appropriate. Data are expressed as mean ± SD and p values reported are 2-sided.

Results

Esomeprazole is cytotoxic to human melanoma in vitro independently from the mutational profile

We evaluated the anti-tumor activity of PPI on human melanoma cells and whether PPI effectiveness was related to molecular features. Mutational analysis and protein expression profiles performed in the melanoma cell lines showed heterogeneity in expression level and mutation rate of genes involved in melanoma pathogenesis and cell death pathways (Table 1). Moreover, since PPI target subunit A of human V-ATPase[25] and subunit a expression is involved in tumor cell invasion,[37] we evaluated the expression of subunit A (peripheral domain, V1) and subunit a (integral domain, V0) in the melanoma cell lines (Table 2). Interestingly, the expression of V-ATPase subunit a was significantly higher in metastatic

Table 1. Analysis of protein expression and genetic mutations in the melanoma cell lines

	Protein expression						Mutational analysis					
Cell line	pAKT	AKT	pERK	ERK	PTEN	p53	BRAF	NRAS	PTEN	p16	p14	p53
Me30966	++	+	++	+	−	++	V600E	wt	C105fsX112	HD (exon 1 α, 2, 3)	HD (exon 2)	R175H
Me20842	−	+	+	+	+	+	L597S	wt	wt	wt/+	wt/+	G187S
Me9923	+	+	+	+	+	++	wt	G12S	wt	wt/−*	wt/+	wt
Me5810	+	+	++	+	−	+	wt	Q61R	wt	wt/−	wt/− *	wt
Me15392	−	+	−	+	+	++	V600E	wt	wt	HD (exon 1 α, 2, 3)	HD (exon 1 β, 2)	wt
Mel501	−	+	++	+	−	+	wt	G10D[a]	ND	HD (exon 1 α, 2, 3)	HD (exon 1 β, 2)	wt
Me30631	+	+	+	+	−	+	wt	wt	wt	HD (exon 1 α, 2, 3)	HD (exon 1 α, 2, 3)	wt
WM902	+	+	+	+	+	++	V600E	wt	ND	A60fs	G75fs	Y220C
WM793	−	+	++	+	+	+	V600E	wt	ND	wt	wt	wt
Me2658	−	+	++	+	+	++	V600E	ND	ND	HD (exon 1 α, 2, 3)	HD (exon 1 β, 2)	wt

HD, homozygosis deletion; wt, wild-type gene; ND, not done; −, absence of protein; +, presence of protein; ++, protein overexpression; *, absence of mRNA.

Table 2. The inhibitory effect of ESOM on proliferation and the expression level of V-ATPase (subunits A and a) in 10 melanoma cell lines cultured at different pH are shown

Cell line	IC_{50} (μM) pH 7.4	IC_{50} (μM) Unbuffered	IC_{50} (μM) pH 6.0	V-ATPase Sub A	V-ATPase Sub a
Me30966 (M)	112 ± 13	55 ± 4	21 ± 2	33 ± 2	84 ± 4
Me20842 (P)	130 ± 10	95 ± 9	62 ± 6	27 ± 2	41 ± 3
Me9923 (P)	85 ± 9	71 ± 8	63 ± 6	31 ± 2	60 ± 3
Me5810 (P)	142 ± 12	95 ± 8	39 ± 6	38 ± 3	91 ± 5
Me15392 (M)	117 ± 12	83 ± 7	30 ± 4	74 ± 5	145 ± 9
WM902 (P)	124 ± 15	103 ± 8	95 ± 11	35 ± 2	79 ± 4
Mel501 (M)	74 ± 8	50 ± 4	28 ± 3	58 ± 3	140 ± 9
WM793 (P)	173 ± 18	106 ± 11	95 ± 9	28 ± 1	91 ± 4
Me2658 (M)	52 ± 4	25 ± 2	14 ± 2	23 ± 1	90 ± 5
Me30631 (M)	57 ± 6	50 ± 8	31 ± 4	43 ± 3	95 ± 4
Mean IC_{50}	107 ± 12	73 ± 8	48 ± 9	39 ± 5	92 ± 10

IC_{50} is expressed as mean ± standard errors; intracellular expression of V-ATPase subunit a and A was detected by FACS and expressed as mean fluorescence intensity; P and M indicate whether the cell lines derived from Primary or Metastatic lesions.

cells (111 ± 13 MFI) as compared to primary cells (72 ± 10 MFI, $p < 0.05$).

On the basis of these observations, we evaluated the cytotoxic effects of ESOM on melanoma cells. Since PPI are prodrugs which need acidity to be transformed in the active molecule, we reasoned that tumors should be sufficiently acidic to induce proper PPI activation. Human melanoma cells, like other tumor cells of different origin are able to grow *in vitro* unaffected by acidity (Supporting Information Fig. 1*a*), as it occurs *in vivo*.[8,9] Thus, we investigated the antiproliferative effect of PPI in buffered and acidic culture conditions (*i.e.*, unbuffered medium and medium at pH 6.0). Most experiments were run by using omeprazole and esomeprazole with comparable results, so data with esomeprazole (ESOM) are reported. ESOM treatment induced a dose-dependent inhibition of cell proliferation in all culture conditions (Table 2). ESOM IC_{50} was significantly lower in unbuffered and even lower in pH 6.0 medium as compared to buffered medium ($p < 0.05$) (Table 2). Consistent with the increased expression of subunit a, metastatic melanoma showed increased sensitivity to ESOM as compared to cells derived from primary lesions. Moreover, the ESOM effect was even higher in metastatic cells cultured at pH 6.0 (Supporting Information Fig. 1*b*). Analysis of cell viability by MTS assay performed on some cell lines showed that ESOM induced dose-dependent decrease in cell viability (Fig. 1*a*) and dramatic morphological changes in melanoma cells as represented for Me30966 cells in Figure 1*b*. These results were confirmed and supported by experiments in which the cytotoxic effect of ESOM was evaluated by SRB assay to measure cell survival in additional metastatic melanoma cell lines (Supporting Information Fig. 1*c*).

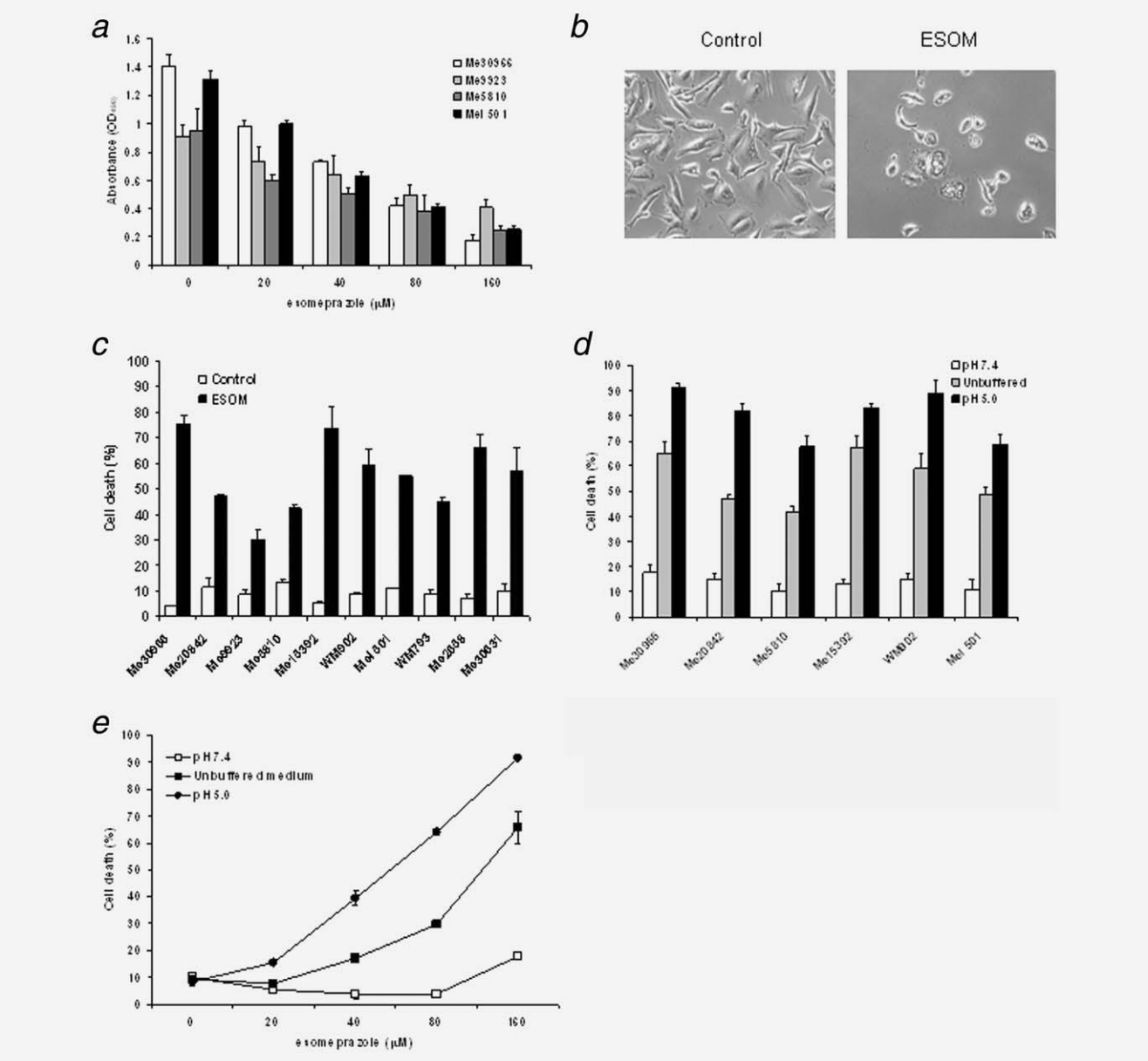

Figure 1. ESOM induces pH-dependent cell death in melanoma cells. (*a*) ESOM induces dose-dependent loss of cell viability (MTS assay) in melanoma cells cultured in unbuffered medium for 24 hr. Experiments were run in triplicates and repeated twice. (*b*) Phase-contrast image of Me30966 cells untreated or treated for 24 hr with ESOM (160 μM). (*c*) ESOM (160 μM) induces cell death in human melanoma cells cultured in unbuffered medium for 24 hr. Cell death was defined as the percentage of Annexin-V+ cells. (*d*) The cell death induced by ESOM (160 μM) is enhanced by acidic culture medium and undetectable in cells cultured in neutral pH medium. (*e*) Quantification of cell death in presence of ESOM in different pH culture conditions is representatively shown for Me30966 cells. To note, cells cultured in buffered medium (pH 7.4) are not sensitive to ESOM-induced cytotoxicity.

Esomeprazole induce pH- and caspase-dependent cell death in human melanoma

To analyze the ESOM-induced cytotoxic effect, we cultured melanoma cell lines in unbuffered medium in the presence of ESOM (160 μM) for 24 hr. Cell death was determined as the percentage of Annexin-V+ cells, including both PI− and PI+ cells. The analysis showed that ESOM caused cell death in all melanoma cells tested (Fig. 1*c*), independently of their genetic background and signaling pathway alterations. The appearance of dead cells was detectable as early as 8 hr after treatment because of the presence of Annexin-V+/PI− cells (not shown). We then investigated the role of pH in ESOM-induced cell death by culturing melanoma cells in unbuffered medium and medium at pH 5.0. We observed that ESOM-induced cell death was significantly increased in cells cultured in medium at pH 5.0 (Fig. 1*d*) while no cytotoxic effect was

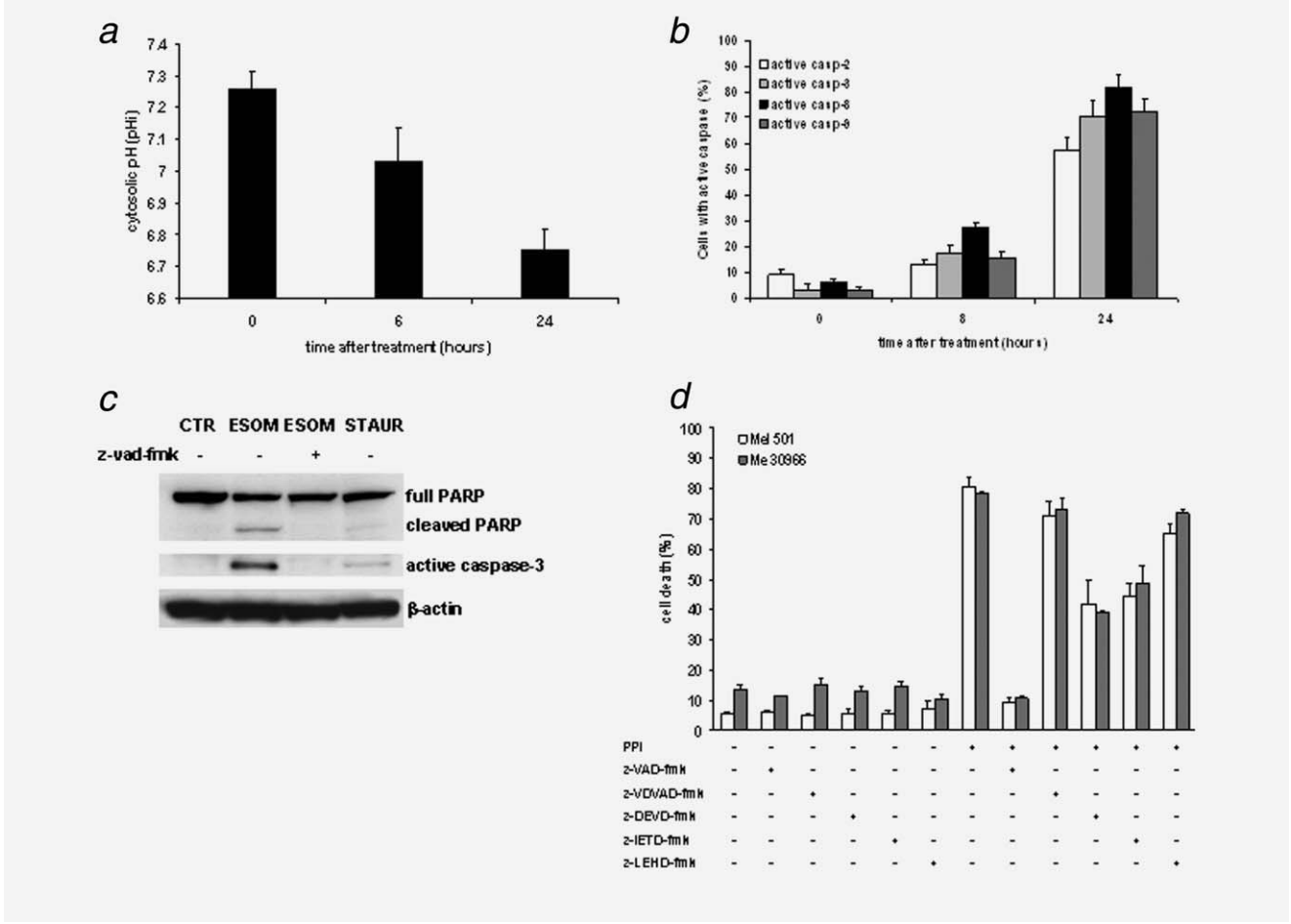

Figure 2. ESOM induces caspase-dependent cell death in melanoma cells. (*a*) ESOM treatment induces intracellular acidification in melanoma cells (Me30966). (*b*) ESOM treatment of Me30966 cells induces a time-dependent activation of several caspases, including caspase 2, 3, 8 and 9. (*c*) Western blot analysis of active caspase-3 and PARP-1 cleavage following ESOM treatment in Me30966 cells cultured in unbuffered medium in presence or absence of Z-VAD-fmk (10 μM). Adherent and floating cells were used to make the protein lysate. As positive control cells were treated for 18 hours with staurosporine (0.1 μM). (*d*) ESOM-induced cell death in Me30966 and Mel501 cells is completely abrogated by pan-caspase inhibitor Z-VAD-fmk and reduced by caspase-3 and -8 inhibitors. * indicates $p < 0.05$. All experiments were performed culturing cells in unbuffered medium and ESOM was used 160 μM.

detectable at neutral pH. Moreover, as represented for Me30966 cells (Fig. 1*e*), the increased activity of ESOM in low pH culture condition was dose-dependent.

An important mechanism of resistance to cytotoxic stimuli of malignant tumor cells is the ability to maintain an alkaline pHi.[16,17] In fact, we measured the pHi in the panel of melanoma cell lines and found that pHi was clearly alkaline (7.43 ± 0.16). Thus, we cultured Me30966 cells in unbuffered medium in the absence or presence of ESOM (160 μM) and found that ESOM treatment induced a time-dependent intracellular acidification (Fig. 2*a*), consistent with the inhibitory activity of ESOM on V-ATPase, which results in drastic break of proton extrusion into extracellular environment and H+ accumulation within cells.

Intracellular acidification has been shown to modulate caspases activation.[38,39] Interestingly, ESOM-induced cytotoxic effect was accompanied by a time-dependent activation of several caspases as shown for Me30966 cells cultured in unbuffered medium (Fig. 2*b*). To analyze the role of caspases in ESOM-induced cell death the pan-caspases inhibitor Z-VAD-fmk was titrated and used 10 μM, a concentration known to specifically inhibit caspases (Supporting Information Fig. 2*a*). We observed by western blot that preincubation with Z-VAD-fmk inhibited the activation of caspase-3 and the caspase-mediated cleavage of PARP-1 in ESOM-treated Me30966 cells (Fig. 2*c*). The Z-VAD-fmk effect on inhibition of active caspase-3 was also shown by FACS analysis (Supporting Information Fig. 2*b*). In line with these observation, preincubation with Z-VAD-fmk resulted in abrogation of cell death in Me30966 and Mel501 cells (Fig. 2*c* and Supporting Information Figs. 2*a* and 2*c*) and inhibition of active caspase-3 (Supporting Information

Fig. 2*b*). Using caspase-specific inhibitors we could observe that while pan-caspase inhibition completely abrogated cell death, inhibition of caspases 3 and 8 partially reduced such effects (Fig. 2*d*).

Altogether, these data indicate that blockade of proton pumps through ESOM induced caspase-dependent cell death in human melanoma cells. PPI-mediated cytotoxicity was independent from their genetic background, but dependent on extracellular acidity.

ESOM alters tumor pH *in vivo*

We asked whether ESOM might function as antitumor drug *in vivo* where tumors grow in solid mass with a progressive acidification of tumor microenvironment and derangement of pH gradients between the extracellular and the intracellular compartments. We investigated whether ESOM could target the tumor site, thus altering tumor pH as it normally does in the gastric antrum. To this purpose, we engrafted SCID mice with human melanoma cells (Me15392) according with previously published protocol[40] and performed MRS analysis on tumor lesions in untreated and ESOM-treated animals. About 20 days after tumor engraftment (*i.e.*, when lesions reached a mean size of 600 mm^3) untreated mice ($n = 10$) and animals treated with a single dose of ESOM (2.5 mg kg^{-1}) ($n = 10$) were submitted to MRS analysis for tumor pH measurement. ESOM dose was chosen based on pharmacological studies[41] and because roughly corresponding to maximum dosage used in humans (160–240 mg day^{-1}).[29,30] First, we found that pHe of engrafted human melanoma was acidic [6.42 ± 0.19] while pHe of healthy tissues surrounding the tumor ranged between 7.0 and 7.1 ($p < 0.001$), demonstrating that melanoma-engraftment in SCID mice created an acidic environment suitable for testing PPI-induced pH modulation.

Tumor pHe was analyzed at different times after ESOM administration. Alkalinisation of tumor pHe was detectable 3 hr after ESOM treatment, as shown by the chemical shift of 3-APP signal in MRS spectra of untreated *vs.* treated (ESOM 2.5 mg kg^{-1}) melanoma lesions (Fig. 3*a*). Evaluation of long-term effects of ESOM indicated that tumor pHe increased within 5 hr, and returned to pretreatment values 48 hr after ESOM administration (Fig. 3*b*). Overall, ESOM induced a significant increase of tumor pHe that shifted toward neutrality [6.77 (6.58–6.96)] (Fig. 3*c*). Consistent with *in vitro* results showing ESOM-induced intracellular acidification (Fig. 2*a*), *in vivo* ESOM treatment caused acidification of tumors pHi (Fig. 3*c*). Notably, as a consequence of these effects, the pH gradient across the plasma membrane of tumor cells (ΔpH = pHi − pHe) significantly decreased upon ESOM treatment [0.24 (0.04–0.44)] with respect to untreated tumors [0.68 (0.60–0.76)], getting close the ΔpH characterizing healthy tissues (Fig. 3*c*). Similar results were observed in mice engrafted with the cell line Me5810 (Supporting Information Fig. 3*a*).

These data provided the proof of concept that ESOM targets tumor site *in vivo* and exerts its expected effect on tumor pH gradients, providing a rationale for its potential antineoplastic activity.

ESOM reduces tumor growth *in vivo*

To assess the antineoplastic role of PPI *in vivo*, we tested whether ESOM administration could affect the growth of human melanoma engrafted in SCID mice. This model was chosen as particularly suited for evaluating direct effects of drugs on human tumors, without possible interference from host cell components. ESOM administration schedule was designed based on *in vivo* evidence that tumor pHe displayed an initial shift towards neutrality after ESOM treatment, returning to basal values within 48 hr (Fig. 3*b*). Since acidic pHe is needed for PPI to be transformed in the active drug, we established a treatment protocol consisting of 6 ESOM doses given orally every other day. The dose of 2.5 mg kg^{-1}, roughly comparable to maximum dosage administered to patients with Zollinger-Ellison syndrome (160–240 mg day^{-1}),[42] was tested in comparison with 0.5 and 0.1 mg kg^{-1}. Seven days after injection of melanoma cells (Me15392), when tumors were resent as palpable lesions (~200 mm^3), ESOM was administered and tumor growth was monitored for 2–4 weeks. Potential side effects of high dosage ESOM were monitored in treated animals and none of treatments was associated with significant toxicity at any organ level. Indeed, treated mice were monitored for the duration of experiments for body weight, hair ruffling and presence of diarrhoea and showed no sign of illness.

In line with *in vitro* data on the cytotoxic effects of ESOM on human melanoma cells, ESOM administration significantly reduced melanoma *in vivo* growth in a dose-dependent manner (Fig. 4*a*). The most relevant anti-tumor effect was achieved using ESOM 2.5 mg kg^{-1}, providing ~50% decrease of melanoma lesion size as compared with control animals ($p = 0.01$) while a 30% reduction was obtained with 0.5 mg kg^{-1} ($p = 0.05$). Indeed, ESOM (2.5 mg kg^{-1}) mediated a 50% reduction of tumor growth with lesions reaching 1211 ± 202 mm^3 as compared with 2322 ± 322 mm^3 of tumor mass developing in untreated animals (Fig. 4*a*). Similar results were observed using Mel501 cells (Fig. 4*b*), with tumor size reaching 836 ± 91 mm^3 in control group and 435 ± 101 mm^3 in treated animals ($p = 0.004$). Interestingly, comparable ESOM anti-tumor effects were obtained in mice engrafted with melanoma cell lines Me15392, Mel501 and Me5810 (Supporting Information Fig. 3*b*) characterized by different genetic mutations and diverse signaling pathways.

Immunohistochemical analysis of Mel501 melanoma lesions revealed the presence of large necrotic areas in ESOM-treated tumors with respect to untreated animals (Fig. 4*c*). Moreover, tumors from ESOM-receiving mice were characterized by decreased proportion of Ki-67+ cells (Fig. 4*c*), suggesting a reduced proliferative potential of residual tumor cells. These results suggest that ESOM cytotoxicity on tumor cells is paralleled by concomitant inhibition of the proliferative rate within the tumor mass.

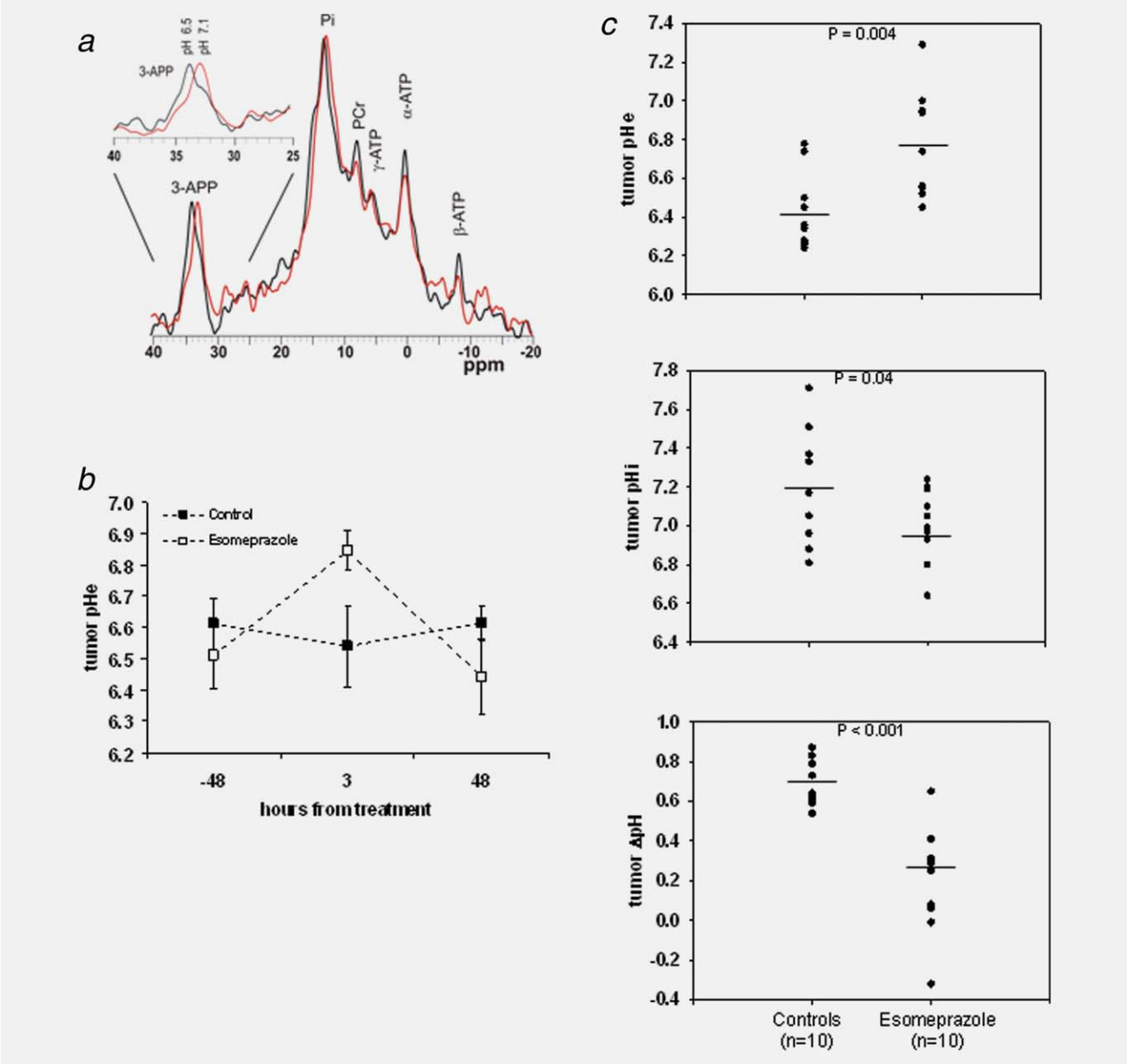

Figure 3. Effects of ESOM treatment on tumor pH *in vivo*. (*a*) ^{31}P MRS *in vivo* spectra from a subcutaneous melanoma (Me15392) 1 day before (black trace) and 3 hr after (red trace) ESOM administration (2.5 mg kg^{-1}). (*b*) Tumor pHe in melanoma lesions before and after ESOM administration (2.5 mg kg^{-1}). (*c*) Effects of ESOM treatment on tumor pHe, pHi and ΔpH (pHi-pHe) are shown in melanoma lesions from untreated and treated SCID mice. Dots represent individual values and lines represent mean values.

ESOM increases survival of SCID mice engrafted with human melanoma

To evaluate the effects of long-term ESOM treatment, we delivered ESOM intraperitoneally to avoid animal stress due to either repeated gavages or intravenous administration. Mice were engrafted with Mel501 cells and 7 days later ESOM (2.5 and 12.5 mg kg^{-1}) was administered for 6 weeks with 2 different treatment schedules, consisting of either 3 consecutive days/week or 3 times/week every other day. Mice engrafted with Mel501 cells usually show severe toxicity in terms of weight loss and morbidity within 30 days. The 2 treatment schedules showed substantially similar results in terms of tumor growth and animal survival, so data from pooled animals are reported. Long-term ESOM treatment resulted in a dose-dependent reduction of tumor growth until untreated animals were alive (Fig. 5*a*). Moreover, during the observation period the animals treated with 12.5 mg kg^{-1} ESOM experienced a reduced weight loss (1.2 ± 0.4 g), as

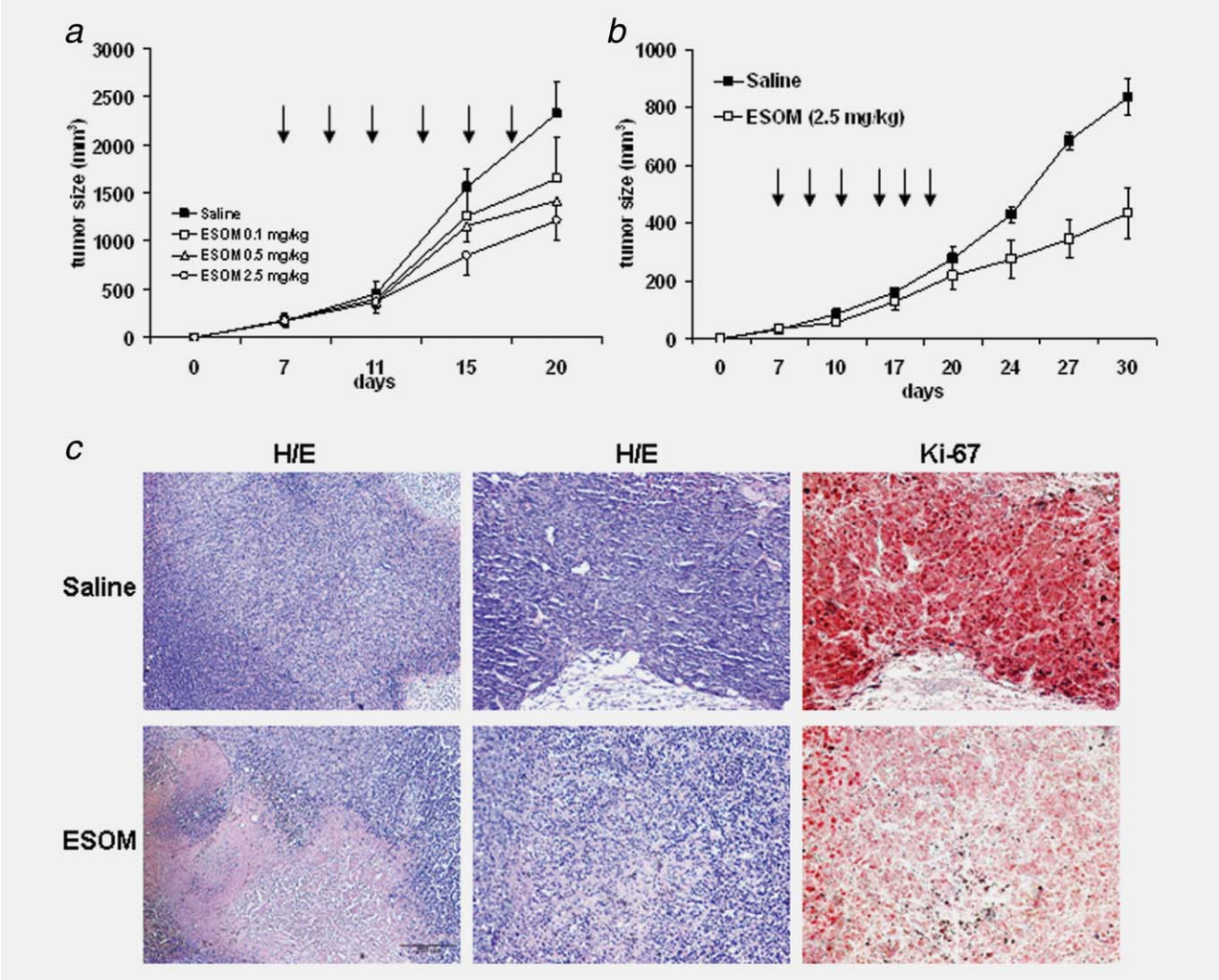

Figure 4. Effects of ESOM treatment on melanoma growth *in vivo*. Inhibitory effect of ESOM treatment on growth of Me15392 cells (*a*) and Mel501 cells (*b*). In these experiments, each group consisted of 8 animals and ESOM was administered by gavage every other day starting from day 7 for a total of 6 administrations. The presence of large necrotic areas is observed in ESOM treated melanoma (Mel501) *in vivo* (*c*). In Mel501-derived tumors treated with 2.5 mg kg^{-1} ESOM the tumor mass is occupied by a large necrotic area (left column). Serial sections of the same tumors shows diffused necrosis (middle column) and decreased proliferating (Ki-67+) tumor cells (left column).

compared to untreated animals (2.6 ± 0.2 g) (Fig. 5*b*), supporting the beneficial effects of treatment. The median survival time of untreated animals was 24 days while for animals treated with ESOM 2.5 mg kg^{-1} the median survival was significantly longer (45 days, Fig. 5*c*). Intriguingly, in the group of mice treated with ESOM 12.5 mg kg^{-1}, 80% of animals survived and remained symptoms-free during the observation period, without reporting any signs of systemic toxicity.

These data indicate that long-term ESOM treatment not only reduces growth of human melanoma but dramatically improves animal survival.

Discussion

The present study provides the first preclinical evidence that high dosage of PPI administration induces remarkable cytotoxicity in human melanoma cell lines and reduces growth of human melanoma in SCID mice, without evidence of systemic toxicity. The inhibition of tumor growth observed in human melanoma xenografts was consistent with a dramatic increase of animal survival, supporting new strategy for cancer therapy based on the effective and safe use of this class of drugs.

Tumor cell metabolism is being nowadays object of renewed consideration for better understanding of cancer biology and therapeutic strategies.[2,43] The adaptive response to metabolic stress occurring in cancer cells includes indeed the upregulation of proton extrusion,[2,43,44] which represents a detoxification mechanism significantly contributing to tumor microenvironment acidification.[2] We showed in previous reports that inhibition of proton pumps by PPI impairs

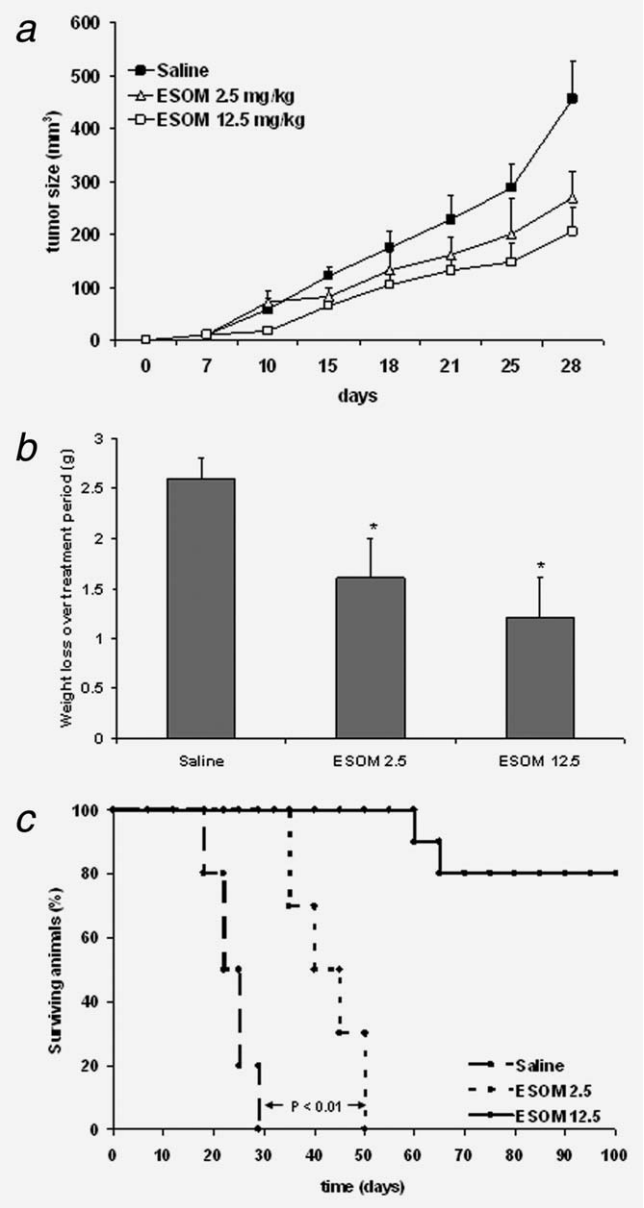

Figure 5. Effects of ESOM treatment on tumor growth, weight and survival of human melanoma-bearing animals. Mice ($n = 20$ for each group) were engrafted with Mel501 cells and ESOM (2.5 and 12.5 mg kg^{-1}) was administered *via* i.p. 3 times/week for 6 weeks. (*a*) Tumor growth was monitored until all animals in untreated group were dead. (*b*) The absolute weight loss (weight at Day 7—weight at Day 28) is shown for the 3 groups of mice and * indicates $p < 0.05$. (*c*) Survival rate of untreated and ESOM-treated human melanoma-engrafted SCID mice.

viability of human B cell tumors and sensitizes drug-resistant melanoma cells to chemotherapeutics.[14,26] Since PPI are pro-drugs acting only at acidic pH, we reasoned that altering tumor pH regulation through proton pump inhibition would selectively impair tumor growth without affecting metabolism of normal tissues. In fact, low pH culture condition highly potentiated the inhibition of proliferation and the induction of cell death by the PPI prototype ESOM in human melanoma cells. It has been reported that acidic environment induces p53-dependent apoptosis in cancer cells, thus mutations in p53-dependent apoptotic pathways may reduce acid-mediated toxicity.[45] However, we observed that the cytotoxic effect of ESOM was not related to particular mutational profile or activated signalling pathways, suggesting that PPI may encompass molecular hallmarks of human melanoma.

Cell lines derived from metastatic lesions showed increased expression of V-ATPase subunit a as compared to primary cells, together with an increased sensitivity to the antiproliferative effects of PPI at low pH, suggesting that proton pumps activity may represent a key mechanism for homeostatic adaptation of metastatic cells to adverse microenvironment conditions as recently reported.[13,21–23,46,47] This observation is also in line with recent reports on the crucial role of V-ATPase in invasion and metastasis of cancer cells.[13,19,21,37]

Reversal of pH gradient across plasma membrane and membranes of intracellular organelles is an important hallmark of malignancy,[3,4,15,48] related to invasion, metastasis,[13,15] proliferation and resistance to chemotherapy.[16,49,50] In fact, we found that ESOM induced acidification of the pHi both *in vitro* and *in vivo*, thus creating the optimal conditions for caspase activation,[16] and killing melanoma cells through a caspase-dependent mechanism. Interestingly, we recently reported that PPI induce caspase-independent cell death with involvement of mitochondrial membrane depolarization in human lymphoblastic tumors despite the presence of activated caspases,[26] suggesting that these drugs may trigger different cell death pathways depending on tumor type and pHe of tumors. In fact, it should be underlined that ESOM-induced melanoma cell death in this experimental setting induced also typical features of caspase-mediated cell death like PARP cleavage and DNA fragmentation (data not shown) but it did not occur through mitochondria alterations (data not shown), as the lack of caspase-2 involvement also suggests. Interestingly, it has been reported that mitochondria may be not required for cytosolic acidification during cell death[51] and that cytosolic acidification requires active caspases.[52,53] In line with these observations, we found that the presence of z-vad-fmk partially inhibited the cytosolic acidification induced by ESOM in melanoma cells (Supporting Information Fig. 2*d*).

Recently, the concept of targeting or exploiting the acidic tumor pHe as an antitumor therapeutic strategy has been supported by several studies.[4,31,54–58] While the alkalinisation of tumor pHe with bicarbonate was shown to inhibit metastasis formation and enhance chemotherapy,[57,59] the use of pH-sensitive lytic peptides and nanotechnology have been suggested to selectively target tumor tissue.[55,60] However, our study provides the proof of concept that pro-drugs like PPI may function as antitumor drugs exploiting the tumor acidic pHe both as a therapeutic target and also as a selective

delivery system.[3,4,26] We demonstrate by *in vivo* MRS analysis that orally administered ESOM targets the tumor site, shifting the baseline tumor pHe from acidity to neutrality and reducing tumor pHi, thus globally affecting pH gradients. This may have important consequences not only on the capacity of malignant cells to proliferate and survive in acidic conditions but also on distribution and penetration of chemotherapeutics in tumor microenvironment and tumor cells.[3,5,48] Consistent with the derangement of tumor pH gradients, ESOM clearly inhibited tumor growth and dramatically prolonged survival of melanoma-bearing animals.

Moreover, our results provide new preclinical information on possible treatment doses and schedule. PPI, as efficient inhibitors of proton pumps are widely used for treatment of peptic diseases with minimal side effects.[28] We first show that an effective dose of ESOM is 2.5 mg kg^{-1}, that is perfectly compatible with doses administered daily to patients with Zollinger-Ellison syndrome,[29,30] receiving up to 240 mg day^{-1} ESOM for several days without major side-effects.[28,42] MRS data on tumor pH following ESOM administration suggested that a possible schedule for ESOM treatment of human patients could be discontinuous administration of 2.5 mg kg^{-1}. However, we also observed that daily ESOM treatment had comparable effect on tumor growth inhibition and significantly increased animal survival. Based on this information, Phase II clinical trials using esomeprazole as single drug for treatment of metastatic melanoma patients are ongoing in Italy.

Recent literature supports the concept that PPI delivery is not restricted to the stomach, but also to other acidic compartments such as skin in the case of vitiligo patients[61] and patients affected by cutaneous leishmaniasis.[62] This suggested that ESOM may trigger vitiligo, through an inhibitory effect on melanosomes maturation (Supporting Information Fig. 2*e*) and/or the induction of oxidative stress in vitiligo melanocytes[61,63] but it may as well target the skin exerting its antileishmanial activity. Moreover, new studies strongly indicate that PPI may also interact with other molecular targets, explaining their diverse potential clinical effects.[64,65]

In conclusion, our study adds important information on the pre-clinical setting-up for the use of PPI in the treatment of a poorly treatable tumor such as human melanoma. We provide evidence that PPI may induce a direct anti-tumor effect, without interference with possible triggering of systemic reactions and with a specific delivery to the tumor site.

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