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

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

Brief statements: **This work demonstrates that proton pump inhibitors induce a pH-mediated and caspase-dependent cell death in human melanoma cells through modifying tumor pH gradients. We propose that targeting tumor pH regulation by proton pump inhibitors may represent a novel and feasible therapeutic strategy against human melanoma.**

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Abstract

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 pro-drugs 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 signalling 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) 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 pre-clinical 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.

Introduction

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 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, favours 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 pre-clinical study [14] represented the rationale for two 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 pre-clinical 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.

Materials and methods

Drugs

Omeprazole and esomeprazole sodium salts (AstraZeneca, Sweden) were resuspended 2 mg/ml 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 characterised [35], melanoma cell lines Mel501, WM902 and WM793 (a gift from Dr. Meenhard Herlyn, 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 hours 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 hours 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 hours 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 hours prior the addition of ESOM. Pepstatin A (Roche Molecular Biochemicals, Milano), CA-074 (Calbiochem, Milano) and E64d (Sigma Aldrich) were respectively used 100 μM, 10 μM 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 x 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 one wash in Wash Buffer the cells were analysed 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 analysed 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 Signalling). After probing with HRP-conjugated anti-mouse or anti-rabbit antibodies (Cell Signalling) 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 x 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 x width^2 /2. In one 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) was administered by gavage. The 3-APP probe (128 mg/kg) was administered i.p. immediately prior to MRI/MRS analyses. Additional technical details are provided.

A Varian Inova 200/183 MRI/MRS system for small animals operating at 4.7 T was used for MRI/MRS analysis. A 1cm two-turn or a 2cm three-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 ms, α= 20°, thickness=2 mm, 8 averages, FOV= 3x3 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=2000/23ms). ^{31}P localized spectra were acquired from the tumor with ISIS (TR/TM= 2000/80 ms, 2048 averages, VOI ranging from 300 to 400 µl) or ^{1}H-decoupled pulse-acquire sequence (TR= 3000 ms, α=25°, garp, 256 averages).

Statistical analysis

Differences between groups were analysed by Mann-Whitney test, student T test or by ANOVA as appropriate. Data are expressed as mean ± SD and P values reported are two-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 cells (111±13 MFI) as compared to primary cells (72±10 MFI, $P < 0.05$).

Based on these observations, we evaluated the cytotoxic effects of ESOM on melanoma cells. Since PPI are pro-drugs 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 Figure 1A), 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 Figure 1B).

Analysis of cell viability by MTS assay performed on some cell lines showed that ESOM induced dose-dependent decrease in cell viability (Figure 1A) and dramatic morphological changes in melanoma cells as represented for Me30966 cells in Figure 1B. 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 Figure 1C).

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

In order to analyse the ESOM-induced cytotoxic effect, we cultured melanoma cell lines in unbuffered medium in the presence of ESOM (160 μM) for 24 hours. 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 (Figure 1C), independently of their genetic background and signalling pathway alterations. The appearance of dead cells was detectable as early as 8 hours 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 (Figure 1D) while no cytotoxic effect was detectable at neutral pH. Moreover, as represented for Me30966 cells (Figure 1E), 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 (Figure 2A),

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 (Figure 2B). 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 Figure 2A). 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 (Figure 2C). The Z-VAD-fmk effect on inhibition of active caspase-3 was also shown by FACS analysis (Supporting information Figure 2B). In line with these observation, pre-incubation with Z-VAD-fmk resulted in abrogation of cell death in Me30966 and Mel501 cells (Figure 2C and supporting information Figure 2A,C) and inhibition of active caspase-3 (Supporting information Figure 2B). 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 (Figure 2D).

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) (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) [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 7.0-7.1 ($P < 0.001$), 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 hours 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) melanoma lesions (Figure 3A). Evaluation of long-term effects of ESOM indicated that tumor pHe increased within 5 hr, and returned to pre-treatment values 48 hours after ESOM administration (Figure 3B). Overall, ESOM induced a significant increase of tumor pHe that shifted towards neutrality [6.77 (6.58-6.96)] (Figure 3C). Consistent with *in vitro* results showing ESOM-induced intracellular acidification (Figure 2A), *in vivo* ESOM treatment caused acidification of tumors pHi (Figure 3C). Notably, as a consequence of these effects, the pH gradient across the plasma membrane of tumor cells ($\Delta pH = pHi - pHe$) 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 □pH characterizing healthy tissues (Figure 3C). Similar results were observed in mice engrafted with the cell line Me5810 (Supporting information Figure 3A).

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 hours (Figure 3B). 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, roughly comparable to maximum dosage administered to patients with Zollinger-Ellison syndrome (160-240 mg/day) [42], was tested in comparison with 0.5 and 0.1 mg/kg. 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 (Figure 4A). The most relevant anti-tumor effect was achieved using ESOM 2.5 mg/kg, providing approximately 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 (P = 0.05).

Indeed, ESOM (2.5 mg/kg) 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 (Figure 4A). Similar results were observed using Mel501 cells (Figure 4B), 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 Figure 3B) characterized by different genetic mutations and diverse signalling pathways.

Immunohistochemical analysis of Mel501 melanoma lesions revealed the presence of large necrotic areas in ESOM-treated tumors with respect to untreated animals (Figure 4C). Moreover, tumors from ESOM-receiving mice were characterized by decreased proportion of Ki-67+ cells (Figure 4C), 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.

ESOM increases survival of SCID mice engrafted with human melanoma

To evaluate the effects of long-term ESOM treatment, we delivered ESOM intraperitoneally in order to avoid animal stress due to either repeated gavages or intravenous administration. Mice were engrafted with Mel501 cells and seven days later ESOM (2.5 and 12.5 mg/kg) was administered for 6 weeks with two 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 two 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 (Figure 5A). Moreover, during the observation period the animals treated with 12.5 mg/kg ESOM experienced a reduced weight loss (1.2 ± 0.4 g), as compared to untreated animals (2.6

± 0.2 g) (Figure 5B), 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 the median survival was significantly longer (45 days, Figure 5C). Intriguingly, in the group of mice treated with ESOM 12.5 mg/kg, 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 pre-clinical 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 up-regulation 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 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 Figure 2D).

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, that is perfectly compatible with doses administered daily to patients with Zollinger-Ellison syndrome [29, 30], receiving up to 240 mg/day 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. 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 Figure 2E) 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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References

1. Korn EL, Liu PY, Lee SJ, Chapman JA, Niedzwiecki D, Suman VJ, Moon J, Sondak VK, Atkins MB, Eisenhauer EA, Parulekar W, Markovic SN, et al. Meta-analysis of phase II cooperative group trials in metastatic stage IV melanoma to determine progression-free and overall survival benchmarks for future phase II trials. J Clin Oncol 2008;26:527-34.

2. Hsu PP, Sabatini DM. Cancer cell metabolism: Warburg and beyond. Cell 2008;134:703-7.

3. De Milito A, Fais S. Tumor Acidity, Chemoresistance and Proton Pump Inhibitors. Future Oncology 2005;1:779-86.

4. Fais S, De Milito A, You H, Qin W. Targeting vacuolar H+-ATPases as a new strategy against cancer. Cancer Res 2007;67:10627-30.

5. Tredan O, Galmarini CM, Patel K, Tannock IF. Drug resistance and the solid tumor microenvironment. J Natl Cancer Inst 2007;99:1441-54.

6. Gatenby RA, Gillies RJ. A microenvironmental model of carcinogenesis. Nat Rev Cancer 2008;8:56-61.

7. Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res 1989;49:6449-65.

8. Negendank W. Studies of human tumors by MRS: a review. NMR Biomed 1992;5:303-24.

9. Griffiths JR. Are cancer cells acidic? Br J Cancer 1991;64:425-7.

10. Becelli R, Renzi G, Morello R, Altieri F. Intracellular and extracellular tumor pH measurement in a series of patients with oral cancer. J Craniofac Surg 2007;18:1051-4.

11. Raghunand N, Gillies RJ. pH and chemotherapy. Novartis Found Symp 2001;240:199-211; discussion 65-8.

12. Morita T, Nagaki T, Fukuda I, Okumura K. Clastogenicity of low pH to various cultured mammalian cells. Mutat Res 1992;268:297-305.

13. Martinez-Zaguilan R, Seftor EA, Seftor RE, Chu YW, Gillies RJ, Hendrix MJ. Acidic pH enhances the invasive behavior of human melanoma cells. Clin Exp Metastasis 1996;14:176-86.

14. Luciani F, Spada M, De Milito A, Molinari A, Rivoltini L, Montinaro A, Marra M, Lugini L, Logozzi M, Lozupone F, Federici C, Iessi E, et al. Effect of proton pump inhibitor pretreatment on resistance of solid tumors to cytotoxic drugs. J Natl Cancer Inst 2004;96:1702-13.

15. Rofstad EK, Mathiesen B, Kindem K, Galappathi K. Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. Cancer Res 2006;66:6699-707.

16. Hirpara JL, Clement MV, Pervaiz S. Intracellular acidification triggered by mitochondrial-derived hydrogen peroxide is an effector mechanism for drug-induced apoptosis in tumor cells. J Biol Chem 2001;276:514-21.

17. Coss RA, Storck CW, Daskalakis C, Berd D, Wahl ML. Intracellular acidification abrogates the heat shock response and compromises survival of human melanoma cells. Mol Cancer Ther 2003;2:383-8.

18. Nishi T, Forgac M. The vacuolar (H+)-ATPases--nature's most versatile proton pumps. Nat Rev Mol Cell Biol 2002;3:94-103.

19. Martinez-Zaguilan R, Lynch RM, Martinez GM, Gillies RJ. Vacuolar-type H(+)-ATPases are functionally expressed in plasma membranes of human tumor cells. Am J Physiol 1993;265:C1015-29.

20. Sennoune SR, Bakunts K, Martinez GM, Chua-Tuan JL, Kebir Y, Attaya MN, Martinez-Zaguilan R. Vacuolar H+-ATPase in human breast cancer cells with distinct metastatic potential: distribution and functional activity. Am J Physiol Cell Physiol 2004;286:C1443-52.

21. Lu X, Qin W, Li J, Tan N, Pan D, Zhang H, Xie L, Yao G, Shu H, Yao M, Wan D, Gu J, 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 2005;65:6843-9.

22. You H, Jin J, Shu H, Yu B, Milito AD, Lozupone F, Deng Y, Tang N, Yao G, Fais S, Gu J, Qin W. Small interfering RNA targeting the subunit ATP6L of proton pump V-ATPase overcomes chemoresistance of breast cancer cells. Cancer Lett 2009.

23. Forgac M. Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology. Nat Rev Mol Cell Biol 2007;8:917-29.

24. Mattsson JP, Vaananen K, Wallmark B, Lorentzon P. Omeprazole and bafilomycin, two proton pump inhibitors: differentiation of their effects on gastric, kidney and bone H(+)-translocating ATPases. Biochim Biophys Acta 1991;1065:261-8.

25. Moriyama Y, Patel V, Ueda I, Futai M. Evidence for a common binding site for omeprazole and N-ethylmaleimide in subunit A of chromaffin granule vacuolar-type H(+)-ATPase. Biochem Biophys Res Commun 1993;196:699-706.

26. De Milito A, Iessi E, Logozzi M, Lozupone F, Spada M, Marino ML, Federici C, Perdicchio M, Matarrese P, Lugini L, Nilsson A, Fais S. Proton pump inhibitors induce apoptosis of human B-cell tumors through a caspase-independent mechanism involving reactive oxygen species. Cancer Res 2007;67:5408-17.

27. Yeo M, Kim DK, Kim YB, Oh TY, Lee JE, Cho SW, Kim HC, Hahm KB. Selective induction of apoptosis with proton pump inhibitor in gastric cancer cells. Clin Cancer Res 2004;10:8687-96.

28. Der G. An overview of proton pump inhibitors. Gastroenterol Nurs 2003;26:182-90.

29. Metz DC, Forsmark C, Lew EA, Starr JA, Soffer EF, Bochenek W, Pisegna JR. Replacement of oral proton pump inhibitors with intravenous pantoprazole to effectively control gastric acid hypersecretion in patients with Zollinger-Ellison syndrome. Am J Gastroenterol 2001;96:3274-80.

30. Ramdani A, Mignon M, Samoyeau R. Effect of pantoprazole versus other proton pump inhibitors on 24-hour intragastric pH and basal acid output in Zollinger-Ellison syndrome. Gastroenterol Clin Biol 2002;26:355-9.

31. Tannock IF, Rotin D. Acid pH in tumors and its potential for therapeutic exploitation. Cancer Res 1989;49:4373-84.

32. Ohta T, Arakawa H, Futagami F, Fushida S, Kitagawa H, Kayahara M, Nagakawa T, Miwa K, Kurashima K, Numata M, Kitamura Y, Terada T, et al. Bafilomycin A1 induces apoptosis in the human pancreatic cancer cell line Capan-1. J Pathol 1998;185:324-30.

33. Nishihara T, Akifusa S, Koseki T, Kato S, Muro M, Hanada N. Specific inhibitors of vacuolar type H(+)-ATPases induce apoptotic cell death. Biochem Biophys Res Commun 1995;212:255-62.

34. Gallagher FA, Kettunen MI, Day SE, Hu DE, Ardenkjaer-Larsen JH, Zandt R, Jensen PR, Karlsson M, Golman K, Lerche MH, Brindle KM. Magnetic resonance imaging of pH in vivo using hyperpolarized 13C-labelled bicarbonate. Nature 2008;453:940-3.

35. Daniotti M, Oggionni M, Ranzani T, Vallacchi V, Campi V, Di Stasi D, Torre GD, Perrone F, Luoni C, Suardi S, Frattini M, Pilotti S, et al. BRAF alterations are associated with complex mutational profiles in malignant melanoma. Oncogene 2004;23:5968-77.

36. Morton DB, Griffiths PH. Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. Vet Rec 1985;116:431-6.

37. Hinton A, Sennoune SR, Bond S, Fang M, Reuveni M, Sahagian GG, Jay D, Martinez-Zaguilan R, Forgac M. Function of a subunit isoforms of the V-ATPase in pH homeostasis and In Vitro invasion of MDA-MB231 human breast cancer cells. J Biol Chem 2009.

38. Matsuyama S, Llopis J, Deveraux QL, Tsien RY, Reed JC. Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. Nat Cell Biol 2000;2:318-25.

39. Marches R, Vitetta ES, Uhr JW. A role for intracellular pH in membrane IgM-mediated cell death of human B lymphomas. Proc Natl Acad Sci U S A 2001;98:3434-9.

40. Lozupone F, Pende D, Burgio VL, Castelli C, Spada M, Venditti M, Luciani F, Lugini L, Federici C, Ramoni C, Rivoltini L, Parmiani G, et al. Effect of human natural killer and gammadelta T cells on the growth of human autologous melanoma xenografts in SCID mice. Cancer Res 2004;64:378-85.

41. Katashima M, Yamamoto K, Sugiura M, Sawada Y, Iga T. Comparative pharmacokinetic/pharmacodynamic study of proton pump inhibitors, omeprazole and lansoprazole in rats. Drug Metab Dispos 1995;23:718-23.

42. Shi S, Klotz U. Proton pump inhibitors: an update of their clinical use and pharmacokinetics. Eur J Clin Pharmacol 2008;64:935-51.

43. Kroemer G, Pouyssegur J. Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell 2008;13:472-82.

44. Provent P, Benito M, Hiba B, Farion R, Lopez-Larrubia P, Ballesteros P, Remy C, Segebarth C, Cerdan S, Coles JA, Garcia-Martin ML. Serial in vivo spectroscopic nuclear magnetic resonance imaging of lactate and extracellular pH in rat gliomas shows redistribution of protons away from sites of glycolysis. Cancer Res 2007;67:7638-45.

45. Williams AC, Collard TJ, Paraskeva C. An acidic environment leads to p53 dependent induction of apoptosis in human adenoma and carcinoma cell lines: implications for clonal selection during colorectal carcinogenesis. Oncogene 1999;18:3199-204.

46. Lugini L, Matarrese P, Tinari A, Lozupone F, Federici C, Iessi E, Gentile M, Luciani F, Parmiani G, Rivoltini L, Malorni W, Fais S. Cannibalism of live lymphocytes by human metastatic but not primary melanoma cells. Cancer Res 2006;66:3629-38.

47. Torigoe T, Izumi H, Ise T, Murakami T, Uramoto H, Ishiguchi H, Yoshida Y, Tanabe M, Nomoto M, Kohno K. Vacuolar H(+)-ATPase: functional mechanisms and potential as a target for cancer chemotherapy. Anticancer Drugs 2002;13:237-43.

48. Gerweck LE, Vijayappa S, Kozin S. Tumor pH controls the in vivo efficacy of weak acid and base chemotherapeutics. Mol Cancer Ther 2006;5:1275-9.

49. Reshkin SJ, Bellizzi A, Caldeira S, Albarani V, Malanchi I, Poignee M, Alunni-Fabbroni M, Casavola V, Tommasino M. Na+/H+ exchanger-dependent intracellular alkalinization is an early event in malignant transformation and plays an essential role in the development of subsequent transformation-associated phenotypes. Faseb J 2000;14:2185-97.

50. Rich IN, Worthington-White D, Garden OA, Musk P. Apoptosis of leukemic cells accompanies reduction in intracellular pH after targeted inhibition of the Na(+)/H(+) exchanger. Blood 2000;95:1427-34.

51. Waibel M, Kramer S, Lauber K, Lupescu A, Manns J, Schulze-Osthoff K, Lang F, Wesselborg S. Mitochondria are not required for death receptor-mediated cytosolic acidification during apoptosis. Apoptosis 2007;12:623-30.

52. Meisenholder GW, Martin SJ, Green DR, Nordberg J, Babior BM, Gottlieb RA. Events in apoptosis. Acidification is downstream of protease activation and BCL-2 protection. J Biol Chem 1996;271:16260-2.

53. Liu D, Martino G, Thangaraju M, Sharma M, Halwani F, Shen SH, Patel YC, Srikant CB. Caspase-8-mediated intracellular acidification precedes mitochondrial dysfunction in somatostatin-induced apoptosis. J Biol Chem 2000;275:9244-50.

54. Kusuzaki K, Murata H, Matsubara T, Satonaka H, Wakabayashi T, Matsumine A, Uchida A. Review. Acridine orange could be an innovative anticancer agent under photon energy. In Vivo 2007;21:205-14.

55. Lee ES, Gao Z, Bae YH. Recent progress in tumor pH targeting nanotechnology. J Control Release 2008.

56. Raghunand N, He X, van Sluis R, Mahoney B, Baggett B, Taylor CW, Paine-Murrieta G, Roe D, Bhujwalla ZM, Gillies RJ. Enhancement of chemotherapy by manipulation of tumour pH. Br J Cancer 1999;80:1005-11.

57. Robey IF, Baggett BK, Kirkpatrick ND, Roe DJ, Dosescu J, Sloane BF, Hashim AI, Morse DL, Raghunand N, Gatenby RA, Gillies RJ. Bicarbonate Increases Tumor pH and Inhibits Spontaneous Metastases. Cancer Res 2009.

58. Smallbone K, Gavaghan DJ, Gatenby RA, Maini PK. The role of acidity in solid tumour growth and invasion. J Theor Biol 2005;235:476-84.

59. Raghunand N, Mahoney B, van Sluis R, Baggett B, Gillies RJ. Acute metabolic alkalosis enhances response of C3H mouse mammary tumors to the weak base mitoxantrone. Neoplasia 2001;3:227-35.

60. Makovitzki A, Fink A, Shai Y. Suppression of human solid tumor growth in mice by intratumor and systemic inoculation of histidine-rich and pH-dependent host defense-like lytic peptides. Cancer Res 2009;69:3458-63.

61. Schallreuter KU, Kruger C, Wurfel BA, Panske A, Wood JM. From basic research to the bedside: efficacy of topical treatment with pseudocatalase PC-KUS in 71 children with vitiligo. Int J Dermatol 2008;47:743-53.

62. Kochar DK, Saini G, Kochar SK, Sirohi P, Bumb RA, Mehta RD, Purohit SK. A double blind, randomised placebo controlled trial of rifampicin with omeprazole in the treatment of human cutaneous leishmaniasis. J Vector Borne Dis 2006;43:161-7.

63. Namazi MR. Proton pump inhibitors may trigger vitiligo by rendering melanocytes prone to apoptosis. Br J Dermatol 2008;158:844-5.

64. Cortes JR, Rivas MD, Molina-Infante J, Gonzalez-Nunez MA, Perez GM, Masa JF, Sanchez JF, Zamorano J. Omeprazole inhibits IL-4 and IL-13 signaling signal transducer and activator of transcription 6 activation and reduces lung inflammation in murine asthma. J Allergy Clin Immunol 2009.

65. Mullin JM, Gabello M, Murray LJ, Farrell CP, Bellows J, Wolov KR, Kearney KR, Rudolph D, Thornton JJ. Proton pump inhibitors: actions and reactions. Drug Discov Today 2009;14:647-60.

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

Figure legends

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 hours. Experiments were run in triplicates and repeated twice. (*b*) Phase-contrast image of Me30966 cells untreated or treated for 24 hours with ESOM (160 μM). (*c*) ESOM (160 μM) induces cell death in human melanoma cells cultured in unbuffered medium for 24 hours. 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.**

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.**

Figure 3. Effects of ESOM treatment on tumor pH *in vivo*. **(*a*) ^{31}P MRS *in vivo* spectra from a subcutaneous melanoma (Me15392) one day before (black trace) and 3 hours after (red trace)**

ESOM administration (2.5 mg/kg). (*b*) Tumor pHe in melanoma lesions before and after ESOM administration (2.5 mg/kg). (*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.

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 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).**

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) 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 three groups of mice and * indicates P < 0.05. (*c*) Survival rate of untreated and ESOM-treated human melanoma-engrafted SCID mice.**

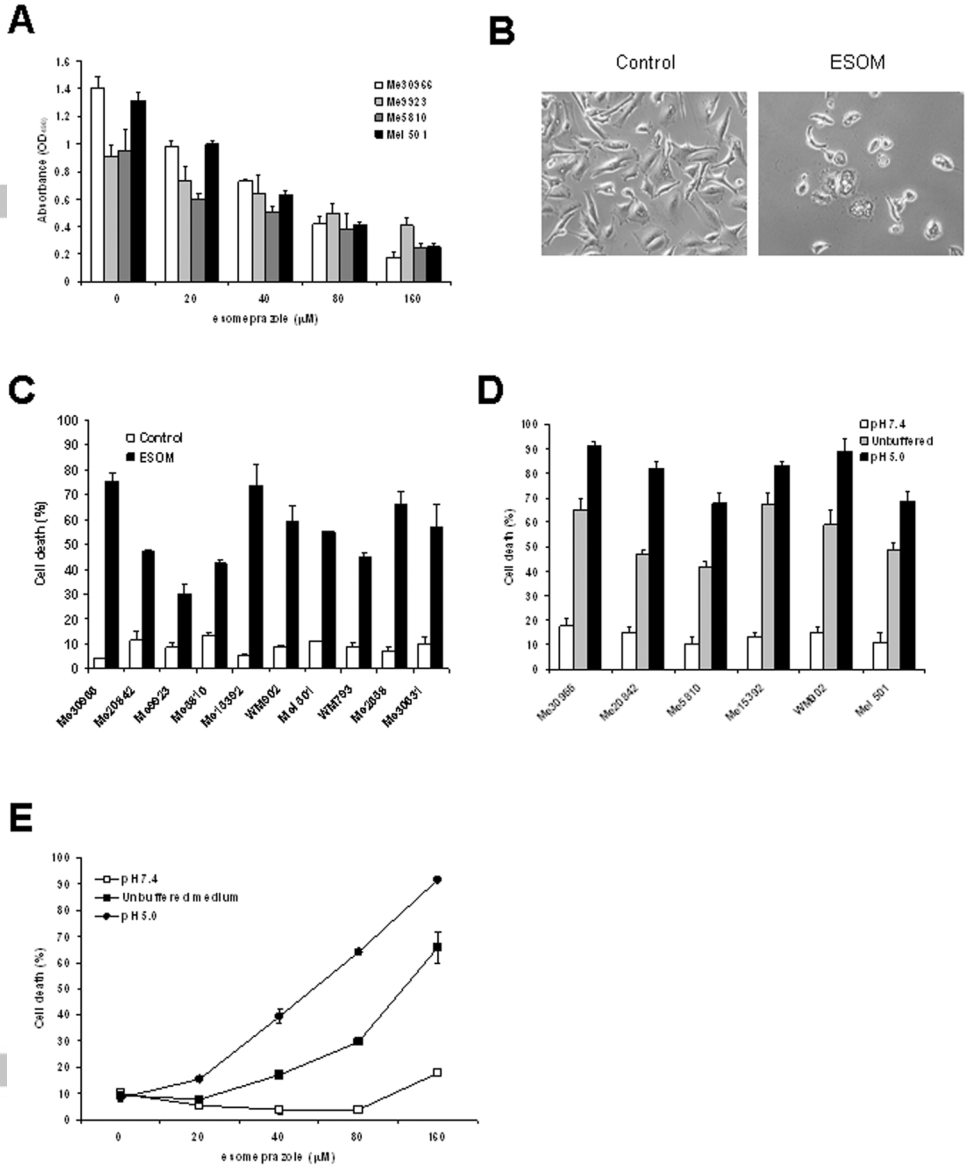

Figure 1

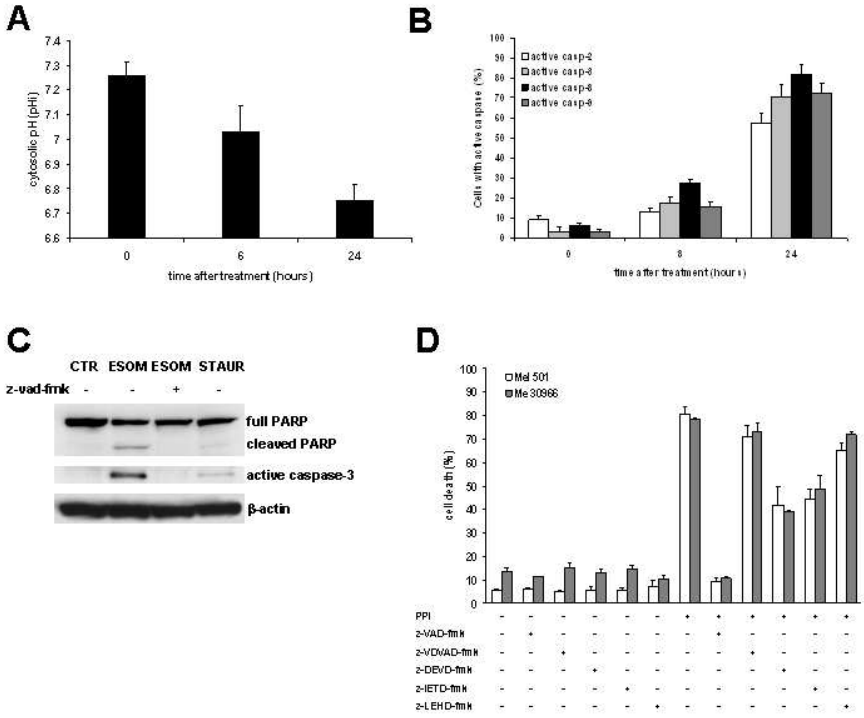

Figure 2

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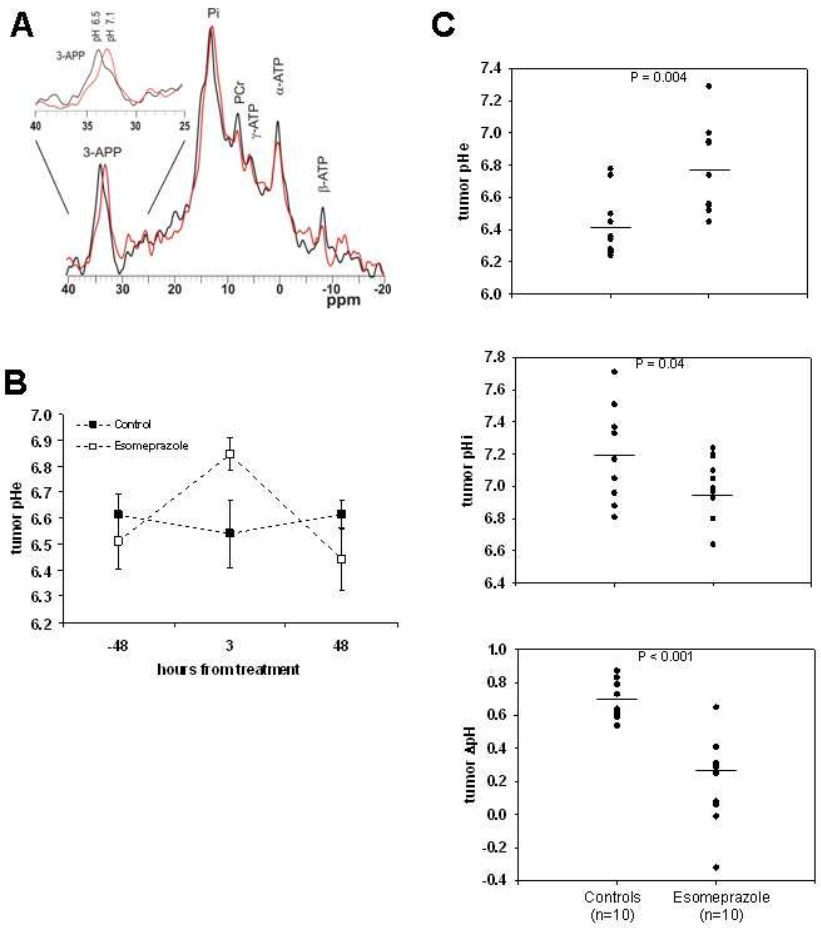

Figure 3

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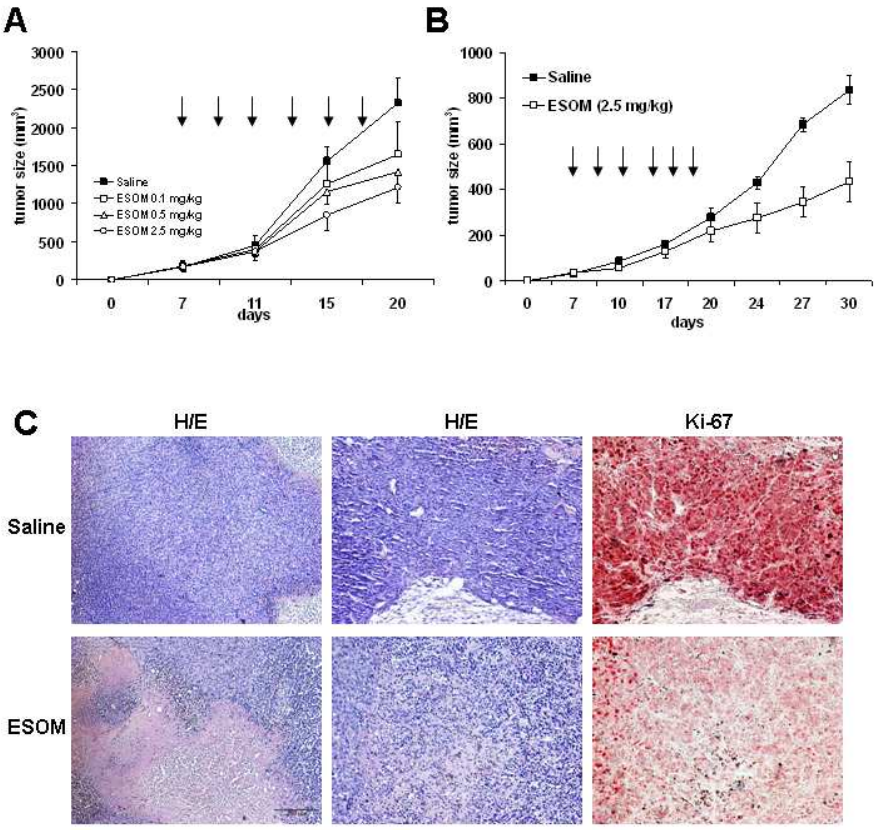

Figure 4

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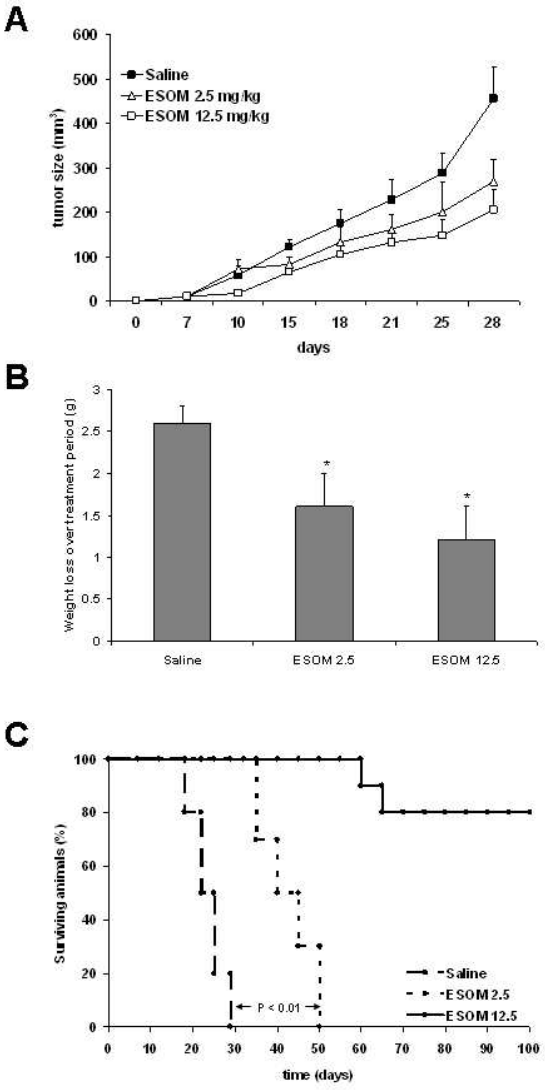

Figure 5

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