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RESEARCH ARTICLE

Lansoprazole and carbonic anhydrase IX inhibitors sinergize against human melanoma cells

Cristina Federici[1], Luana Lugini[1], Maria Lucia Marino[1], Fabrizio Carta[2], Elisabetta Iessi[1], Tommaso Azzarito[1], Claudiu T. Supuran[2], and Stefano Fais[1]

[1]*Department of Therapeutic Research and Medicine Evaluation, National Institute of Health, Rome, Italy and* [2]*NEUROFARBA Department, Sezione di Scienze Farmaceutiche, Università degli Studi di Firenze, Sesto Fiorentino (Florence), Italy*

Abstract

Context: Proton Pump Inhibitors (PPIs) reduce tumor acidity and therefore resistance of tumors to drugs. Carbonic Anhydrase IX (CA IX) inhibitors have proven to be effective against tumors, while tumor acidity might impair their full effectiveness.
Objective: To analyze the effect of PPI/CA IX inhibitors combined treatment against human melanoma cells.
Methods: The combination of Lansoprazole (LAN) and CA IX inhibitors (FC9-399A and S4) has been investigated in terms of cell proliferation inhibition and cell death in human melanoma cells.
Results: The combination of these inhibitors was more effective than the single treatments in both inhibiting cell proliferation and in inducing cell death in human melanoma cells.
Discussion: These results represent the first successful attempt in combining two different proton exchanger inhibitors.
Conclusion: This is the first evidence on the effectiveness of a new approach against tumors based on the combination of PPI and CA IX inhibitors, thus providing an alternative strategy against tumors.

Keywords

Anti-acid drugs, carbonic anhydrase IX inhibitors, lansoprazole, tumor acidity

History

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Introduction

The ability of cancer cells to become resistant to different drugs, a trait known as multidrug resistance (MDR), remains a significant impediment to successful chemotherapy. Several mechanisms are implicated in resistance to antitumor drugs such as drug sequestration, neutralization in acidic organelles or in the acidic extracellular environment, or increased drug extrusion from the cell via a secretory pathway including extracellular elimination through nanovescicles[1–6]. Increasing evidence shows that altered pH regulation in tumor cells is involved in drug resistance. In particular, the extracellular pH of solid tumors is substantially more acidic than that of normal tissues and the acidic pH of the tumor microenvironment may impair the uptake of weakly basic chemotherapeutic drugs[7,8]. Therefore, the inhibition of several proton extrusion mechanisms adopted by malignant cells, represents one promising therapeutic anti-tumor strategy[9]. The abnormal pH gradient characterizing the tumor cells is finely tuned by different ion/proton pumps including the vacuolar ATPase (V-ATPase)[10,11] whose expression and activity are enhanced in human tumors[12,13]. Inhibition of V-ATPase activity can be achieved by treatment with proton pump inhibitors (PPIs)[14,15], a class of drugs including esomeprazole, omeprazole, lansoprazole, pantoprazole and rabeprazole, currently used in the treatment of peptic diseases and, originally described as specific blockers of gastric H^+-K^+ ATPases[16–19]. They are weak base prodrugs that easily penetrate cell membranes and concentrate in acidic compartments, where they are converted into sulfonamide forms, representing the active proton pump inhibitors[20].

Interestingly, the absence of toxicity of PPIs is largely due to their dependance on the acidic pH for activation, differently to the vast majority of the drugs, including standard anticancer drugs. The protonation in an acidic environment leads to activation instead of neutralization[21].

Our group has extensively investigated PPIs for their potential to reduce tumor acidity and overcome the acid-related chemoresistance. A number of studies have now shown that PPIs can be useful in modulating tumor acidification and restoring chemotherapeutic sensitivity in drug-resistant cancer cells in *in-vitro* and *in-vivo* preclinical studies[8,22–27]. Actually, comparable results were obtained by a molecular knock down of V-ATPase subunits[28], just supporting a key role of these proton pumps in drug resistance of tumors.

Specific cytotoxic effects of PPIs on tumor cells have been reported as well, including B cell lymphoma[29], melanoma[30], pancreatic cancer[25], esophageal cancer[31], gastric carcinoma[32], Ewing sarcoma[33], osteosarcoma, rhabdomyosarcoma and chondrosarcoma[34,35]. These preclinical data have been supported by clinical studies in both patients with osteosarcoma[34] and breast

Address for correspondence: Stefano Fais, Department of Therapeutic Research and Medicine Evaluation, National Institute of Health, Viale Regina Elena, 299, 00161 Rome, Italy. Tel: +39.06.49903195. Fax: +39.06.49902436. E-mail: stefano.fais@iss.it

cancer[26] and in companion animals with spontaneous tumors[36,37].

Recently, we compared both in *in-vitro* and *in-vivo* different members of the PPIs family in order to investigate which was the most suitable among the PPIs for the treatment of cancer patients. In fact, despite PPIs belong to the same class of generic drugs, they have different chemical features. The results showed that Lansoprazole (LAN) was the most effective in terms of cytotoxic effect against metastatic melanoma, osteosarcoma and for the first time against glioblastoma, a well-known chemotherapy refractory tumor[38]. In particular, in human melanoma cells, we have shown that Lansoprazole is very effective in both modulating tumor acidification and enhancing sensitivity to suboptimal doses of Paclitaxel, consistent with a reduction of systemic toxicity[27].

In many types of hypoxic tumors, two isoforms of the metalloenzyme Carbonic Anhydrase (CA) are highly expressed, CA IX and XII, but they lack from normal tissues. They are involved in tumor acidification, metastasis and invasion and their inhibition leads to a profound antitumor effect and are commonly referred to as the tumor-associated isoforms[39]. CA is a family of metalloenzymes that catalyze the rapid conversion of CO_2 to HCO_3^- and H^{+}[40].

CA isoforms are found in a variety of tissues where they participate in several important biological processes such as acid-base balance, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis and electrolyte secretion. Many CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited/activated for the treatment of a range of disorders such as edema, glaucoma, obesity, cancer, epilepsy and osteoporosis[41–45].

Since both V-ATPases and carbonic anhydrases are proton exchangers involved in the tumor pH regulation, and since their inhibition has antitumor efficacy, we wanted to evaluate whether their inhibition using specific compounds has a clear antitumor action. Actually, this is the first attempt to combine two different proton exchangers as a unique antitumor approach. To this purpose, human melanoma cells have been treated with the sulfamates S4 and *p*-nitrophenyl derivative FC9–399A in combination with LAN, resulting in a marked increase of the single agents' cytotoxic effect. These preliminary data could represent the basis for further studies in order to determine the most effective pharmacological and specific strategies in the treatment of cancer.

Methods

Cell lines

Metastatic melanoma cell line Me30966, supplied by Istituto Nazionale per lo Studio e la Cura dei Tumori, (Milan, Italy) was maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics, at 37 °C in humidified 5% CO_2. Experiments were performed in unbuffered medium (without sodium bicarbonate allowing the cells to generate their own pH), in buffered medium (pH = 7.4) and in acidic medium (pH = 6.0) obtained by the addition of 1 M HCl solution. The pH of all cell culture supernatants were estimated by the use of a pH 123 Microprocessor pH Meter (Hanna Instruments, Milan, Italy).

All cell lines were negative for mycoplasma contamination, as routinely tested by modified nested polymerase chain reaction (VenorGeM Kit, Minerva Biolabs, Berlin, Germany).

Chemicals and reagents

Lansoprazole (Astra-Zeneca, Mölndal, Sweden) was resuspended in 20 mM DMSO immediately before use. In combination treatment experiments, cells were pretreated for 24 or 48 h with Lansoprazole and then treated for additional 24 h with FC9–399A and S4.

Trypan blue was from Alexis Biochemicals (Florence, Italy).

Sulfamates S4 and *p*-nitrophenyl derivative FC9–399A were synthesized according to the procedures reported in the literature (see Table 1).

Western blot

Briefly, subconfluent melanoma cells, following different treatments, were lysed in AKT buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1% NP40] with protease inhibitors (10 g/mL aprotinin and 2 mmol/L phenylmethylsulfonyl fluoride).

Thirty micrograms per sample were resolved on 10% acrylamide gel and transferred to Protran BA85 nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Membranes were blocked overnight with 5% dry milk in PBS1X. Blotting was performed employing anti-CA IX (clone 2D3, Novus Biologicals, USA), anti-β-Tubulin (clone 5H1, BD Biosciences, San Jose, CA) and anti-GAPDH (GAR1, Abcam, Cambridge, UK) monoclonal antibodies.

After incubation with appropriate peroxidase-conjugated anti IgG (Amersham Biosciences, Milan, Italy), membranes were revealed by enhanced chemiluminescence (Pierce, Rockford, IL).

Dose–response curves of FC9–399A and S4

Melanoma cells were plated at 2.5×10^5 per well in 24-well plates in 1 ml of buffered RPMI medium. After 24 h, the cells were treated with FC9–399A or S4 at four different concentrations for each drug. After treatment, cells were collected by pooling cells from the medium (i.e., dead cells) and adherent (live) cells obtained by trypsinization. Cells were washed, resuspended in PBS1X and analyzed by cell death assay as below described. All experiments were run in triplicate wells and repeated at least twice.

Cell proliferation assay

Melanoma cells were plated at 1×10^4 cells per well in 96-well plates in buffered RPMI medium. After 24 h, the medium was replaced with fresh, unbuffered RPMI medium and cells were treated with 50 μM LAN for 24 or 48 h. Then, the medium was removed and the cells were treated with 10 or 50 μM FC9–399A and S4 inhibitors for additional 24 h. After treatment, cell proliferation was determined using 4-nitrophenyl phosphate disodium salt hexahydrate tablets (Sigma-Aldrich, Milan, Italy) and the response was evaluated by the 405 nm absorbance measured by a spectrophotometer ELx800 (Bio-Tek Instruments Inc., Swindon, UK). All experiments were run in triplicate wells and repeated at least twice.

Table 1. CA I, II, IX and XII inhibition data with sulfamates FC8–399A and S4 in comparison with AAZ as standard by a stopped-flow CO_2 hydrase assay[46].

	K_I (nM)*				Selectivity ratio
Compound	hCA I	hCA II	hCA IX	hCA XII	hCA II/ hCA IX
FC9–399A	1230	450	6.0	4.0	75.0
S4	5600	546	7.0	2.0	78.0
AAZ	250	12.1	25.3	5.6	0.48

*Means from three different assays. Errors were within ±5 – 10% of the reported values (data not shown).

Cell death assay

Tumor cells were plated at $3–4 \times 10^5$ cells per well in 24-well plates in buffered RPMI medium. After 24 h, the medium was replaced with unbuffered medium. After additional 24 h, cells were treated with 50 μM of LAN for 24 h. Then, cells were collected by pooling them from the medium (i.e., dead cells) and by trypsinization of adherent cells. Cells were washed and resuspended in PBS1X with 0.4% trypan blue 1:1 (vol/vol) diluition and were analyzed by Flow cytometry on a Becton Dickinson FACScalibur using CellQuestPro software (Becton Dickinson System, Milan, Italy). For each sample the total events were acquired in 60 s. All experiments were run in triplicate wells and repeated at least twice.

Statistical analysis

Differences between treatment groups were analyzed by ANOVA One Way and Bonferroni *t*-test. Data are expressed as mean ± SD and p values reported are two-sided. p Values < 0.001 were considered as statistically significant. Statistical analysis was performed with Sigmastat 3.0 software (San Jose, CA).

Results

Acidic condition reduces the effectiveness of CA IX Inhibitors

In a first set of experiments, we evaluated the CA IX expression in Me30966 melanoma cell line. To this purpose, total extracts of Me30966 cultured in buffered and unbuffered medium were analyzed by Western Blot for CA IX expression. The results (Figure 1a) showed a full and paragonable CA IX expression in both experimental conditions as evaluated by densitometric analysis (data not shown). We, then investigated the dose-response cytotoxic effects of FC9–399A and S4 inhibitors as single treatment on Me30966 cells cultured in either buffered or unbuffered medium in order to assess the effect of culture medium acidity on the activity of the two CA IX inhibitors. In fact, the use of unbuffered medium allows spontaneous culture medium acidification by tumor cell[6,29,30]. The results obtained showed that in the unbuffered medium FC9–399A and S4 CA inhibitors exerted a reduced cytotoxic effect (Figure 1(b) and (c), respectively) as compared to the buffered medium.

Actually, the tumor condition highly resembles that of unbuffered medium, thus suggesting that acidification of tumor microenvironment may have a role in reducing the effectiveness of the FC9–399A and S4 CA inhibitors against tumors due probably to their protonation. These results represented the proof of concept of our hypothesis that a combination of PPI and CA IX inhibitors could represent a fruitful approach in cancer treatment.

Lansoprazole increases the effectiveness of FC9–399A and S4 CA IX inhibitors

On the basis of our experimental evidence about the role of Lansoprazole in increasing sensitization of human melanoma cells to the effect of several standard chemotherapeutic drugs[22,27], we performed experiments aimed at evaluating the PPI-induced

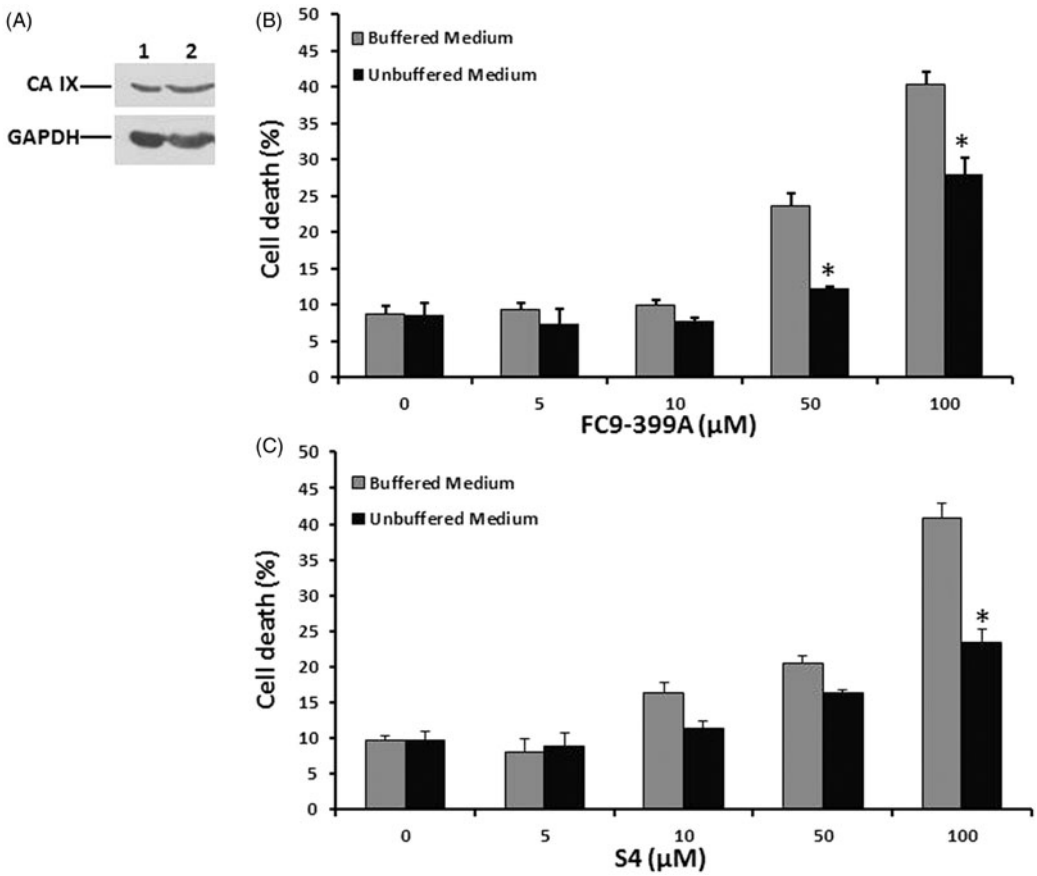

Figure 1. CA IX expression and cytotoxic effect of FC9–399A and S4 inhibitors on Me30966 cells. (a) Western blot analysis of CA IX expression in total cellular extract of Me30966 cultured in unbuffered, line 1 and buffered, line 2 conditions. As a control for protein loading the membranes were blotted with anti-GAPDH antibody (b). Me30966 cells were incubated in buffered and unbuffered medium, treated for 24 h with 5, 10, 50 and 100 μM of FC9–399A (b) and S4 (c). Columns, mean percentages of cell death of three independent experiments run in triplicate; bars indicate SD. (*) indicates $p < 0.001$ (compared to single treatments).

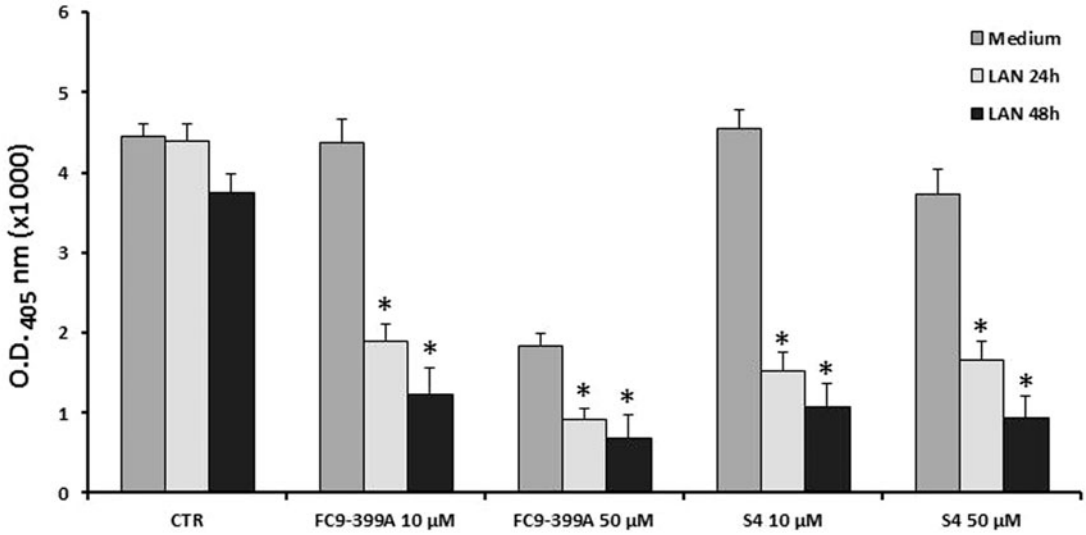

Figure 2. Combined effect of Lansoprazole and FC9–399A or S4 treatment on Me30966 cell growth. Me30966 cells cultured in unbuffered conditions, were treated with LAN for 24 or 48 h. Then, the cells were treated for additional 24 h with FC9–399A or S4 at two different concentrations: 10 and 50 μM. Cell proliferation was evaluated by the 405 nm absorbance measured by a spectrophotometer EL × 800. Columns, mean percentages of cell growth of three independent experiments run in triplicate; bars indicate SD. (*) indicates $p < 0.001$ (compared to single treatments).

sensitization of human melanoma cells to the effect FC9–399A and S4 CA IX inhibitors. For the combined treatment, we used suboptimal doses of both LAN (50 μM) and FC9–399A and S4 CA IX inhibitors (10 μM and 50 μM). Cells were pretreated for 24 and 48 h with LAN and then treated for an additional 24 h with FC9–399A and S4 CA inhibitors. The experiments were performed in unbuffered condition thus mimicking the spontaneous acidification of tumors and allowing the LAN full activation.

Results showed that the 24 h pretreatment with LAN significantly increased the activity of FC9–399A and S4 CA IX inhibitors in melanoma cell line (Figure 2). Fifthy percent tumor growth inhibition was obtained even at only 24 h after LAN pretreatment. Both CA IX inhibitors induced a comparable tumor growth inhibition.

An additional set of experiments were performed to the purpose of evaluating whether the combined treatment passed also through a real cytotoxic effect of tumor cells.

Melanoma cells were pretreated with 50 μM LAN for 24 h and then treated with additional 24 h with FC9–399A or S4 inhibitors at 10 or 50 μM. The results (Figure 3a) showed that the drugs in single treatments induced about 10–15% cell death.

However, LAN pretreatment induced a tumor cell death ranging between 20% at the lower CA IX inhibitors dose, and 40%, at the highest CA IX inhibitors dose, thus demonstrating that this combination was highly effective and dose-dependent against human malignant melanoma cells. A further analysis was aimed to evaluating by western blot whether the combined treatment lead to a reduction of the CA IX expression in treated cells. The results showed that CA IX expression did not change following treatment, thus supporting the hypothesis that the showed effect was due to an inhibition of CA IX activity rather than to a reduction in its expression (Figure 3b).

These results provide strong evidence that LAN pretreatment is highly effective in improving the anti-tumor effect of both FC9–399A and S4 CA IX inhibitors and that this was due to strengthened inhibition of the carbonic anhydrase enzyme activity.

Discussion

During the last decades, pH regulation gained a key role in the development and progression of malignant tumors[47]. The acidic pH of solid tumors has been proposed as a therapeutic target and a drug delivery system for selective anticancer treatments[27,48,49]. Several types of intracellular pH regulatory mechanisms have been identified in tumor cells, among which the V-H^+-ATPases plays a central role[50]. The upregulated proton extrusion activity and lysosomal trafficking confer a selective advantage to tumor cells becoming able to survive in hypoxic-acidic microenvironment[10].

Our previous studies have shown that pretreatment with PPIs consistently induces susceptibility of malignant cells of various histotypes to the cytotoxic effect of different antitumor drugs. This was consistent with a buffering effect on the tumor extracellular milieu and with a marked retention of the chemotherapeutics within the treated tumor cells[22]. We also showed that PPIs have a clear cytotoxic anti-tumor effect when used at high dosages as single agent[29,30]. It is demonstrated that CA IX represents a valuable antitumor target[51–59]. In fact, CA IX has been shown to be upregulated in a number of human cancer tissues as a consequence of either hypoxia-induced or constitutive hypoxia-inducible factor-1 activation, whereas it is not expressed in their normal counterparts, except for gastric mucosa[60]. Although these characteristics make CA IX an interesting target for novel approaches in anticancer therapy, the exact role of CA IX in tumor growth and progression is still unknown. It has been hypothesized that CA IX activity contributes to the environmental acidification of hypoxic tumors through the decrease in extracellular pH[61,62]. Low pH has been associated with tumorigenic transformation, chromosomal rearrangements, extracellular matrix breakdown, tumor cell migration and invasion[62].

Some of us reported preliminary studies towards the evaluation of novel ureido-sulfamate derivatives as potential CA IX inhibitors for the treatment of tumors[63]. Most of the compounds reported showed *in-vitro* an inhibitory effect at low nanomolar concentrations against the tumor associated CA IX and XII[63]. Interestingly, some of them, were able to significantly inhibit the proliferation of SKBR3, MCF10A, ZR75/1, MDA-MB-361 and MCF7 human breast cancer cell lines in both hypoxic and normoxic conditions[63]. Triggered from such encouraging results, herein, we used the best CA IX selective ureido-sulfamate derivatives, such as S4 and FC9–399A, in association with LAN as PPI for their activity against the Me30966 melanoma cell lines (Figure 4 and Table 1).

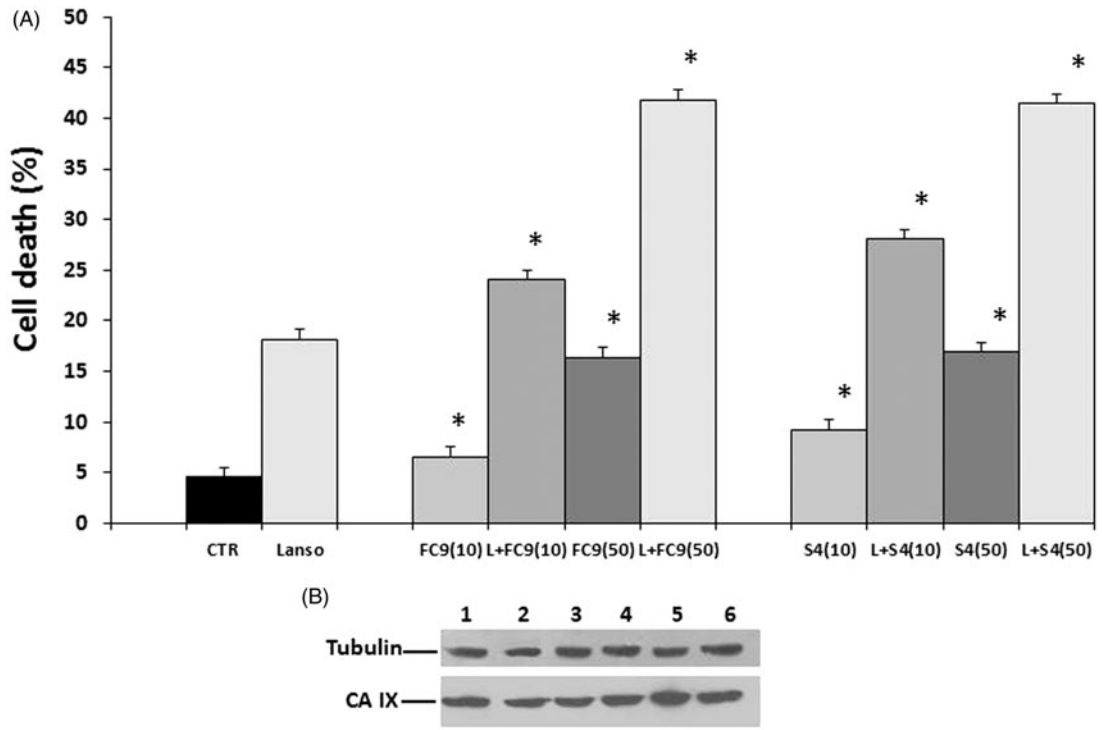

Figure 3. Combined effect of Lansoprazole and FC9–399A or S4 treatment on cell death and CA IX protein expression in Me30966 cells. (a) Me30966 cells cultured in unbuffered conditions, were treated with LAN for 24 h. Then, the cells were treated for additional 24 h with FC9–399A or S4 at two different concentrations: 10 and 50 μM. Tumor cell death was evaluated by Trypan blue exclusion method. Columns, mean percentages of cell death of three independent experiments run in triplicate; bars indicate SD. (*) indicates $p < 0.001$ (compared to single treatments). (b) Western blot analysis of CA IX expression in total cellular extract of (1) Me30966; (2) Me30966 treated with LAN for 24 h; (3) Me30966 treated with FC9–399A for 24 h; (4) Me30966 treated with S4 for 24 h; (5) Me30966 pretreated with LAN and then treated with FC9–399A (50 μM) for additional 24 h; (6) Me30966 pretreated with LAN and then treated with S4 (50 μM) for additional 24 h. As a control for protein loading, the membranes were blotted with anti-tubulin antibody.

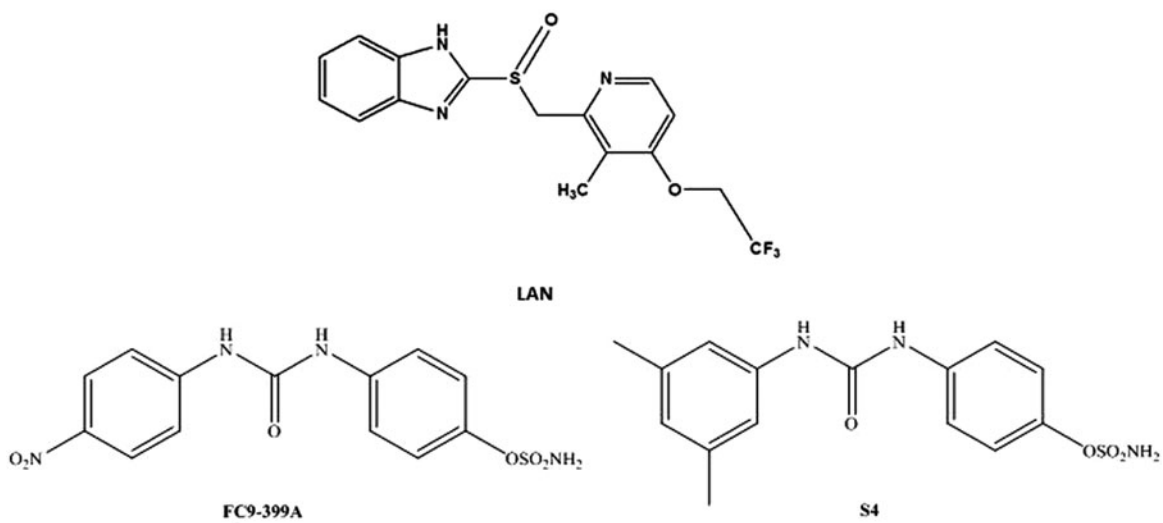

Figure 4. Chemical structure of ureido-sulfamate compounds FC9–399A and S4 and Lansoprazole.

In-vitro kinetic investigations (Table 1) showed that both sulfamate derivatives FC9–399A and S4 were micromolar or high nanomolar inhibitors of the highly abundant off-target CA I and II, whereas they showed low nanomolar K_Is for the tumor associated CA IX and XII. In particular, both compounds were stronger than the classical carbonic anhydrase inhibitor Acetazolamide (AAZ) in inhibiting CA IX (K_Is 6.0, 7.0 and 25.3 nM, respectively), whereas S4 resulted two-fold more potent than FC9–399A against the second tumor associated isoform (K_Is 2.0 and 4.0 nM, respectively).

The results of this study first have shown that CA IX is fully expressed by malignant human melanoma cells. In fact, the use of

specific CA IX inhibitors induced a slight but significant inhibition of melanoma cell growth. However, we postulated that extracellular acidity of melanoma cells might impair the CA IX inhibitors activity due to their protonation outside the cells. Thus, we tested the combination of the proton pump inhibitor Lansoprazole with two different CA IX inhibitors. The results showed that this combination at suboptimal doses induced both tumor cell growth inhibition and a straightforward cytotoxic effect against metastatic melanoma cells, with a significant increase as compared to each single treatment. These results were consistent with the marked reduction of CA IX inhibitors antitumor activity when we cultured melanoma cells in unbuffered medium, known to spontaneously create an acidic extracellular environment. Notably, the spontaneous acidification is suitable for a full activation of PPIs, such as Lansoprazole, and indeed is the real microenvironmental condition of tumors[27,49] and the prime and more efficient mechanism underlying resistance to the vast majority of the anticancer drugs[8]. Moreover, we demonstrated that the effect of combined LAN-CA IX inhibitors treatments did not change the CA IX protein expression, while rather probably inducing an inhibition of the CA IX activity.

Finally, our study further supports the increasing evidence that the tumor acidic microenvironment can be considered as a novel and selective antitumor therapeutic target.

Therefore, the study of new drugs able to counteract the mechanisms involved in the onset tumor acidity, is a priority in the development of new and strategic antitumor therapies.

Conclusions

Tumor acidosis is increasingly considered an important determinant of tumor progression and drug resistance. With this background, a general consensus on the use of a series of proton exchangers' inhibitors in cancer treatment has led to the formation of international society (ispdc)[64] (now iscam), but more importantly to an increasing evidence that there was a panel of inhibitors that might be ready to be used and possibly to be combined in future anti-tumor strategies[9]. However, there was at the same time an obstacle to be overcome: acidosis could represent a neutralizing factor for almost all the proton exchangers' inhibitors, but proton pump inhibitors (PPIs), such as Lansoprazole, that in the acidic microenvironment are transformed into the active molecule[21], and previously proven to counteract tumor resistance to chemotherapeutics[22,27]. This was also supported by results obtained with the knockdown of V-ATPase subunits at the gene levels[28]. In this study, we wanted to explore the hypothesis that PPI could increase the effectiveness of CA IX inhibitors against very malignant human melanoma cells. The results provided the first evidence that combinations of the PPI lansoprazole with two different CA IX inhibitors (FC9–399A and S4) were more effective than single treatments, in inhibiting cell proliferation and inducing cell death in human melanoma cells.

Being tumor acidity, and the expression of both proton pumps and CA IX, common to the vast majority of malignant tumors, our results highly support the use of PPI/CA IX inhibitors as a new antitumor therapeutic approach, with possibly more effectiveness and less toxicity, against all cancers.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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