

## Use of Proton Pump Inhibitors as Adjunct Treatment for Triple-Negative Breast Cancers. An Introductory Study

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**ABSTRACT - PURPOSE:** Triple negative breast cancers (estrogen, progesterone and human epidermal growth factor 2 (HER2) receptor-negative) are among the most aggressive forms of cancers with limited treatment options. Doxorubicin is one of the agents found in many of the current cancer treatment protocols, although its use is limited by dose-dependent cardiotoxicity. This work investigates one of the ways to suppress cancer growth by inhibiting tumor cell ability to remove acid accumulated during its metabolism by proton pump inhibitor esomeprazole (a drug with extensive clinical use) which could serve as an addition to doxorubicin therapy. **METHODS:** In this work, we have investigated growth suppression of triple-negative breast cancer cells MDA-MB-468 by esomeprazole and doxorubicin by trypan blue exclusion assay. Measurement of acidification of treated cancer cells was performed using intracellular pH-sensitive probe, BCECF-AM. Finally, expression of gastric type proton pump ( $H^+/K^+$  ATPase, a target for esomeprazole) on MDA-MB-468 cells was detected by immunofluorescence and Western blotting. **RESULTS:** We have found that esomeprazole suppresses growth of triple-negative breast cancer cell *in vitro* in a dose-dependent manner through increase in their intracellular acidification. In contrast, esomeprazole did not have significant effect on non-cancerous breast epithelial MCF-10A cells. Esomeprazole increases doxorubicin effects suggesting that dual treatments might be possible. In addition, response of MDA-MB-468 cells to esomeprazole could be mediated by gastric type proton pump ( $H^+/K^+$  ATPase) in cancer cells contrary to previous beliefs that this proton pump expression is restricted to parietal cells of the stomach epithelia. **CONCLUSION:** This study provides first evidence that adjunct use of esomeprazole in breast cancer treatment might be a possible to combat adverse effects of doxorubicin and increase its effectiveness.

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### INTRODUCTION

Targeted endocrine therapies have an important role in treatment of breast cancer, though their use is dependent on the presence of specific markers (1). Such therapy is influenced by the tumor's expression of hormonal receptors for estrogen, progesterone and human epidermal growth factor receptor 2 (HER2). In some cases, breast tumors fail to express some or all of those receptors rendering hormone-based treatments ineffective. Triple negative breast cancer (TNBC) is a form of breast cancer characterized by the absence of all three of the hormone receptors (2).

Out of all breast cancer diagnoses each year, more than 15% will represent TNBC phenotype (3). Relative to other forms of breast cancer, TNBC has a more aggressive clinical course, tendency towards visceral metastases and significantly lower survival rate (2). As targeted hormone-based therapies are non-viable options for TNBC, treatments become limited, placing a greater emphasis on standard chemotherapy. A

number of single agents and drug combinations are currently used in TNBC treatment, often to include anthracycline neoplastic antibiotic doxorubicin (DOX).

DOX exerts its pharmacological effects through targeting DNA replication. More specifically, its mechanism includes DNA intercalation and subsequent inhibition of topoisomerase II progression (4). Use of DOX in cancer therapy is limited by its adverse effects, particularly cardiac toxicity including acute, reversible arrhythmias and delayed, irreversible cardiomyopathy (5) even up to 15 years after initial treatment (6). Considering that these adverse effects are dose-related, dose reduction of DOX dose may minimize its toxicity profile. That would only be possible if sensitivity of TNBC to DOX could be increased.

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One potential way to increase such sensitivity could be achieved by manipulating the acid content within tumor cells. There is accumulating evidence suggesting that intracellular pH influences cancer progression and tumor sensitivity to chemotherapy (7). Relative to normal tissue, tumors suffer from hypoxia, increased anaerobic metabolism and as a result create more acidic microenvironment (7). This decrease in intracellular pH leads to the chronic activation of hypoxia-inducible factor-1 (HIF-1), which increases the expression of various glycolytic enzymes (7). The acidic micro-environment of cancer cells have been shown to be correlated with cancer aggressiveness including increased invasiveness, angiogenesis, metastasis and chemo-resistance (8). Factors up-regulated by tumor acid environment include pro-angiogenic factors such as vascular endothelial growth factor (VEGF), interleukin-8, cathepsin B and matrix metalloproteinases-2 and -9, which all enhance tumor invasion and metastatic potential (9, 10). Furthermore, extra-cellular acidity suppresses the activity of cytotoxic T-lymphocytes and natural killer cells consequently decreasing anti-tumor defences (11). Tumors have adapted to acidic micro-environments through over-expression of proton pumps, which extrude protons out from the intra-cellular space of tumor cells. Earlier studies have demonstrated this phenomenon in numerous cell lines, including those derived from gastric cancers, melanomas, colon adenocarcinomas and ovarian adenocarcinomas (12). There are a number of proton pumps over-expressed in malignant tumors. Review by Spugnini *et al* (13) summarizes characteristics of main proton pumps found in mammalian cells. Three of these: vacuolar H<sup>+</sup>-ATPase (expressed on membranes of acidic organelles), Na<sup>+</sup>/H<sup>+</sup>-ATPase (expressed ubiquitously on plasma membranes) and H<sup>+</sup>/K<sup>+</sup> ATPase (expressed in gastric parietal cells) are described as hyper-expressed and/or hyper-functional in malignant tumors (13).

This work addresses the effects of group of drugs collectively known as “proton pump inhibitors” (PPIs, specifically blocking gastric H<sup>+</sup>/K<sup>+</sup> ATPase) on breast cancer cells growth *in vitro*. Structurally, PPIs are specific substituted benzimidazole drugs (esomeprazole, omeprazole, lansoprazole, pantoprazole and rabeprazole among others) developed for suppression of excess acid production in the stomach by irreversibly inhibiting H<sup>+</sup>/K<sup>+</sup>-ATPase in epithelial parietal cells (also known as oxyntic or delomorphous cells). All of the PPIs available

today share that same mechanism of action. They are commonly indicated for a wide range of gastro-intestinal disorders including gastro-oesophageal reflux disease, peptic ulcers and functional dyspepsia (14). Esomeprazole was the first drug to be marketed for various gastropathies and has been proven in regard to its efficacy and tolerability (15).

Considering that the effects of PPIs are restricted to gastric H<sup>+</sup>/K<sup>+</sup>-ATPase (16), it remains unclear which proton pump mediates observed effects in tumor cells of non-gastric origin. To address such discrepancy, vacuolar H<sup>+</sup>-ATPase proton pump with more ubiquitous expression was proposed to be the PPI target in non-gastric tumors (8). Although such possibility may exist, to our knowledge it was never experimentally tested and/or confirmed. Taking into account large structural differences between gastric and vacuolar ATPase pumps and the fact that both respond to their very specific inhibitors (17) we believed that investigation of gastric type proton pump expression in tumor cells of non-gastric origin should be performed in order to explain responses of those cells to PPIs.

Novelty of this work is expressed in its potentially important and previously unreported findings: a) We have shown that PPI esomeprazole suppresses growth of triple-negative breast cancer cells by increasing their intracellular acidity; b) Such action increases tumor cells sensitivity to doxorubicin, a classical anthracycline antibiotic used in many breast cancer treatment protocols; c) In contrast to cancer cells, MCF-10A cells (immortalized but non-transformed human breast epithelial cells) were significantly less sensitive to esomeprazole and d) Effects of esomeprazole appear to be mediated by gastric H<sup>+</sup>/K<sup>+</sup>-ATPase expressed in breast cancer cells.

## METHODS

Unless otherwise indicated, all media, reagents, chemotherapeutic agents listed here were obtained from Sigma-Aldrich (Castle Hill, NSW).

### Cell culture

Triple-negative breast cancer cell line MDA-MB-468 was obtained from American Type Culture Collection. Those cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) foetal bovine serum (FBS) and penicillin/ streptomycin/amphotericin mix, and maintained in a 5% CO<sub>2</sub> humidified incubator at 37°C.

Non-cancerous breast epithelial cell line, MCF-10A, was selected to serve as the control. Although able to grow continuously in culture, MCF-10A cells are positive for epithelial sialomucins, cytokeratins and milk fat globule antigen [18]. MCF-10A cell line was acquired from Prof Andreas Evdokiou (Adelaide University, South Australia). These cells were grown in MEGM Mammary epithelial cell growth medium (Lonza Australia, Mt Waverley VIC) with FBS and antibiotics under the same culture conditions as above.

### Cell treatment

Cells were treated in 6-well plates for 20 h in cell culture incubator in FBS-containing RPMI medium due to its low buffering capacity. After the treatment, cells were washed with PBS and removed from wells by trypsin treatment. Cell suspension was mixed with trypan blue solution and counted using Countess automated cell counter according to manufacturer's suggestions (Invitrogen Australia, Mount Waverley, VIC).

### Measurements of intracellular acidity

Fluorescence of the intracellular pH-sensitive probe, 2',7'-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) (Invitrogen, Mulgrave, VIC), was used to detect relative changes in intracellular pH. After the treatments with either esomeprazole or acidified medium (pH 6.0), cells were washed with HEPES buffered saline followed with incubation with 3  $\mu$ M of BCECF-AM in the same buffer for 30 min at 37°C in the cell culture incubator followed by washing. Fluorescence of intracellular BCECF at 530 nm was detected by Nikon A1-Rsi confocal microscopy. Intensity of fluorescence is reversibly associated with pH. Digital image was semi-quantified to include total fluorescence level and number of cells. The final output given as "relative fluorescence per cell" was compared between treatments.

### Immunofluorescence

Cells were cultured on sterile glass cover slips in 6-well plates at a density of 10,000/cm<sup>2</sup>. After 24 h, cells were rinsed with PBS and fixed with freshly made fixative (4% paraformaldehyde in PBS) for 30 minutes followed by washing with PBS and permeabilized with 0.1% Triton X-100. Immunofluorescence was performed with mouse anti-human H<sup>+</sup>/K<sup>+</sup> ATPase  $\beta$  (C-4) IgG primary antibody and with goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) conjugated secondary antibody (Santa

Cruz, Redcliffe, QLD) and fluorescence detected with Nikon A1-Rsi confocal microscopy.

### Western blotting

Cell lysates were analyzed by Western blotting as we described earlier [19]. H<sup>+</sup>/K<sup>+</sup> ATPase was detected with mouse anti-human H<sup>+</sup>/K<sup>+</sup> ATPase  $\beta$  (C-4) IgG primary monoclonal antibody (Santa Cruz, Redcliffe QLD). Equivalent loading was estimated by Western blotting using mouse anti-human  $\alpha$ -tubulin (B-7) monoclonal antibody (Santa Cruz). Secondary antibody used in these experiments was goat anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad, Gladesville NSW).

### STATISTICAL ANALYSIS

Each measurement is done in triplicates. Data were expressed as mean  $\pm$  standard deviation. In case there were more than two groups of data, they were first analysed by one-way ANOVA. Provided that this significance level was reached, Tukey's multiple comparison post-analysis was used. Unpaired student T tests were used for analysis of data obtained from two data groups (treatment and control). Statistical significance was assigned at  $p < 0.05$ .

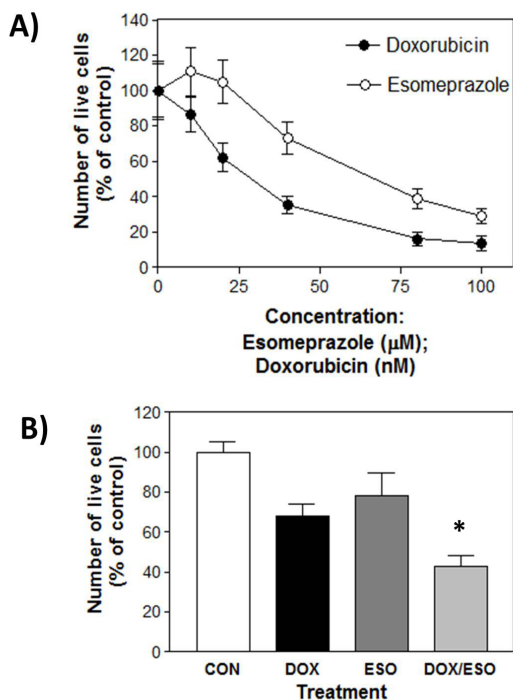
### RESULTS

#### Esomeprazole increases effects of doxorubicin on MDA-MB-468 cells

Treatment of breast cancer MDA-MB-468 cells with both esomeprazole and doxorubicin (Fig. 1A) decreased number of live cells in a dose-dependent manner. As expected, cells were more sensitive to doxorubicin ( $EC_{50} \approx 30$  nM) than esomeprazole ( $EC_{50} \approx 70$   $\mu$ M) (Fig. 1A). In Fig. 1B combinatory treatment of MDA-MB-468 cells with fixed concentrations of esomeprazole (30  $\mu$ M) and doxorubicin (30 nM) is shown. Analysis of the four treatment groups: control (CON), doxorubicin (DOX), esomeprazole (ESO) and combination of doxorubicin an esomeprazole (DOX/ESO) resulted in significant ANOVA outcome ( $P = 0.0004$ ) (Fig. 1B). Post ANOVA analysis revealed significantly improved effects of doxorubicin by esomeprazole compared to doxorubicin alone ( $p < 0.05$ ) (Fig. 1B).

### esomeprazole effects on MDA-MB-468 cells survival is mediated by increase in intracellular acidity

To investigate mechanism of esomeprazole effects on MDA-MB-468 cells we detected changes in intracellular pH in treated cells. For these experiments a pH sensitive intracellular fluorescent probe BCECF-AM was used. BCECF-AM is readily taken up by cells and converted into fluorescent derivative BCECF by cellular esterases. Fluorescence of BCECF is positively correlated with surrounding pH, i.e. as pH decreases so does the BCECF fluorescence. Acidification of MDA-MB-468 cells by either decreasing cell culture medium to pH 6.0 (Fig. 2B) or with the treatment with 50  $\mu$ M esomeprazole (Fig. 2C) decreased relative cells-produced fluorescence compared to controls (Fig. 2A).



**Figure 1.** Sensitivity of MDA-MB-468 cells to esomeprazole and doxorubicin. MDA-MB-468 cells were treated with increasing concentrations of esomeprazole (open circles) or doxorubicin (closed circles) for 20 h. Number of live cells after the treatment was determined by automated trypan blue exclusion assay. Data were expressed relative to control (untreated cells)  $\pm$  SD (all measurements were done in triplicate) (A). MDA-MB-468 cells were treated and effects evaluated as explained above with fixed concentrations of esomeprazole (50  $\mu$ M) and doxorubicin (30 nM) alone (esomeprazole-ESO, doxorubicin-DOX) or in combination (DOX/ESO) (B).

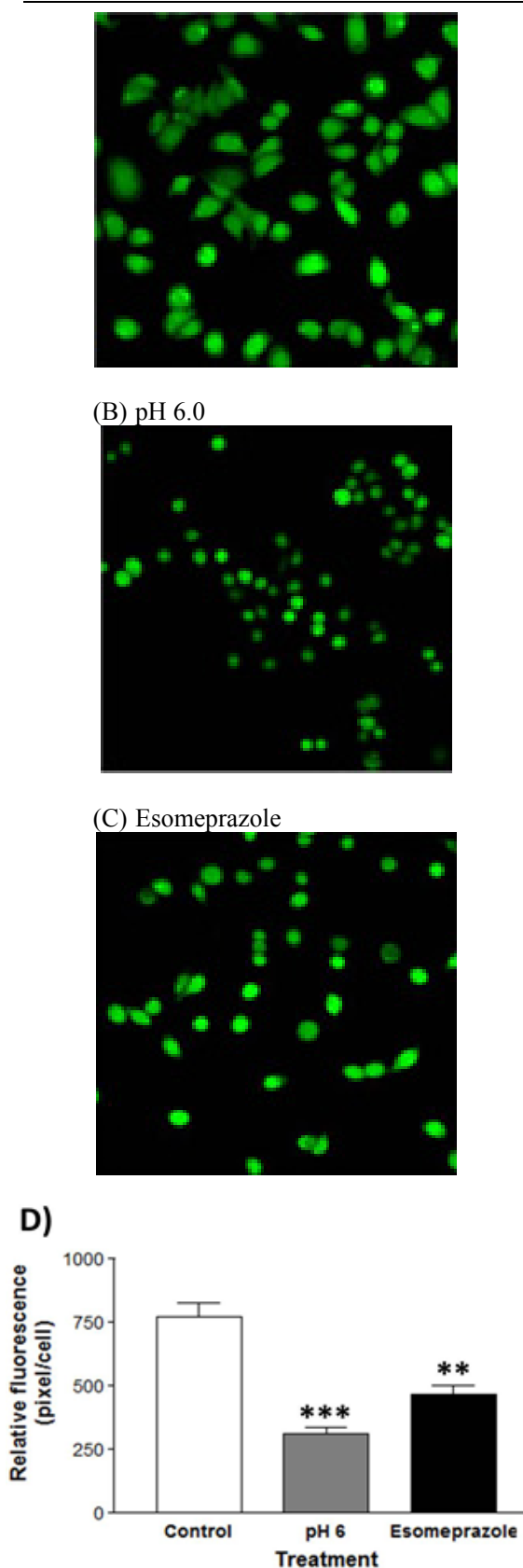
Semi quantitative analysis of detected fluorescence by image analysis is expressed as intensity of green pixels per cell and resulted in significant decrease in intracellular pH after treating cells with 50  $\mu$ M esomeprazole (60.2% of control ( $p < 0.01$  in Tukey's multiple comparison post-analysis) (Fig 2D). Control experiments using cell treatment with medium in which pH has been adjusted to 6.0 produced similar results (decrease in fluorescence to 40.3% of control;  $p < 0.001$ ) (Fig. 2B).

### Increased expression of $H^+/K^+$ -ATPase proton pump in MDA-MB-468 cells is most probably mediating their sensitivity to esomeprazole

Immunofluorescence experiments were used to investigate expression of gastric  $H^+/K^+$ -ATPase proton pump in MDA-MB-468 breast cancer cells and control MCF-10A cells using primary monoclonal antibodies against human gastric  $H^+/K^+$  ATPase  $\beta$  subunit (this proton pump exists as a heterodimer consisting of  $\alpha$  and  $\beta$  subunits). Significantly stronger immunofluorescence was observed with MDA-MB-468 cells compared to control MCF-10A cells (Fig. 3A). Weak immunofluorescence detected in MCF-10A cells was not significantly different from that in control experiments (performed with just a secondary antibody, anti-mouse IgG conjugated with FITC) (data not shown). Expression of gastric pump (33 kDa) in MDA-MB-468 cells was confirmed with Western blotting. Negligible and unreproducible band was detected with MCF-10A cells (Fig. 3B). To indicate equal loading of cell lysate proteins in these experiments, mouse monoclonal antibody raised against  $\alpha$  tubulin (55 kDa) of human origin was used (Fig. 3C). Differences in sensitivity to esomeprazole were also detected in the two cell lines, with MDA-MB-468 cells found to be significantly more sensitive (Fig. 3D). In comparative analysis using fixed concentrations of esomeprazole (100  $\mu$ M) relative number of live MDA-MB-468 cells decreased to approximately 51.88% of control ( $p = 0.014$ , unpaired T test) whereas small decrease in number of treated MCF-10A live cells compared to controls (92.31%) was not statistically significant ( $p = 0.276$ , unpaired T test) (Fig. 3E).

### DISCUSSION

In this work we have investigated increase in sensitivity to doxorubicin of triple-negative breast tumor cells simultaneously treated with esomeprazole and possible mechanism mediating (A) Control



**Figure 2.** Treatment with esomeprazole increases intracellular acidity in MDA-MB-468 cells. MDA-MB-468 cells were treated with either acidified

medium (pH 6.0) (B) or 50  $\mu$ M esomeprazole for 20 h and compared to controls. After the treatment cells were incubated with BCECF-AM followed by fluorescence microscopy image analysis (A and B). Relative fluorescence was estimated by digital image analysis and expressed as intensity of fluorescence per cell (C).

these effects. These findings are potentially important in regard to triple-negative breast cancer treatment that is extremely difficult and usually with limited success.

The findings presented here indicate that combined treatment of TNBC cells with esomeprazole increases their sensitivity to doxorubicin suggesting that dual treatments might be possible.

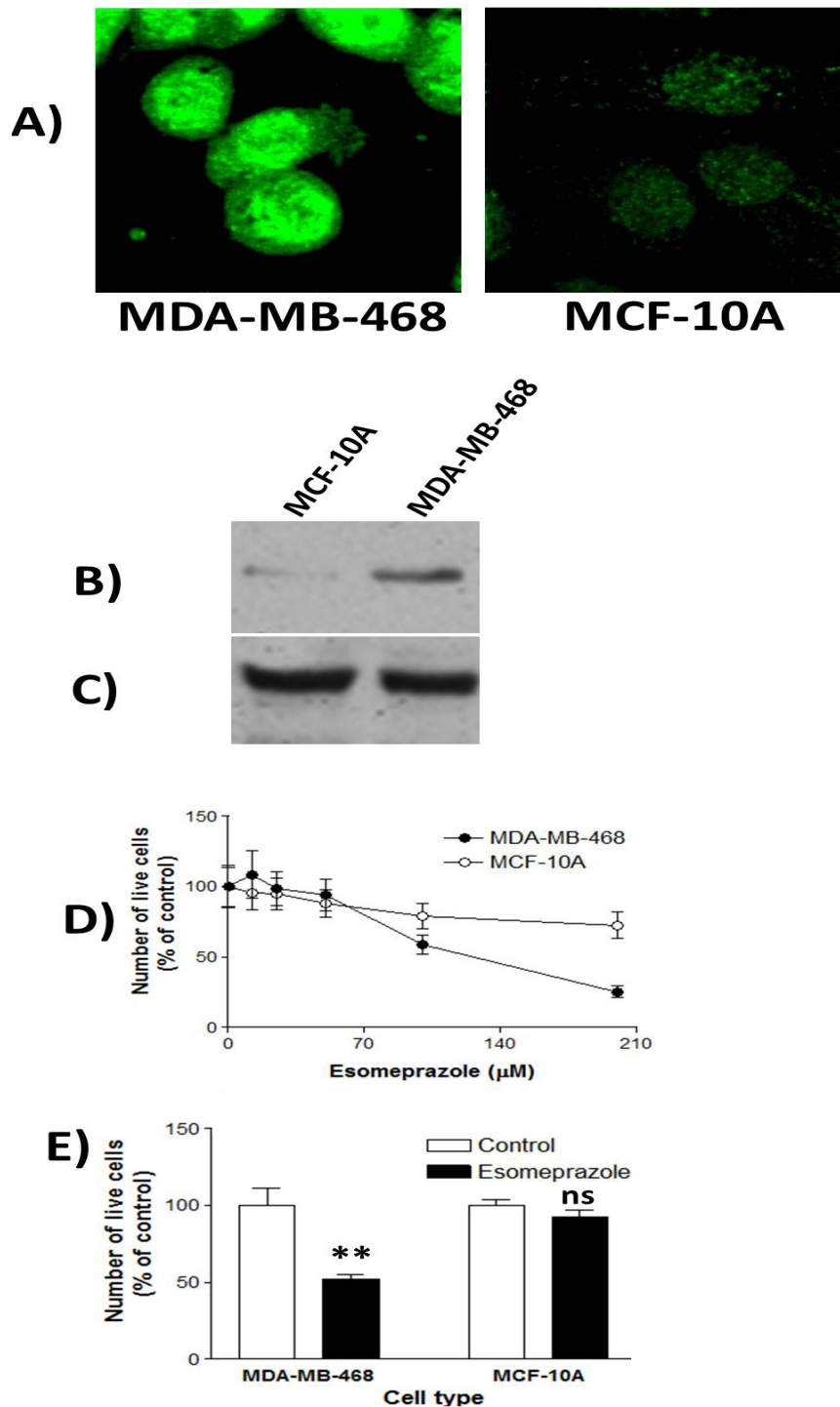
Our data suggest that if PPIs are to be included in future breast cancer treatment protocols, relatively high doses would be needed. This may not be significant problem since PPIs are generally well tolerated with mild and transient side effects including headache and diarrhoea as the most common (20). Furthermore, PPIs have been used in relatively high doses of up to 240 mg IV daily (standard doses range from 10-40 mg), with good tolerability in Zollinger-Ellison syndrome (21). Understanding of the pathophysiology of tumor acidification and adaptive measures of up-regulating proton pumps could be important in future development of new breast cancer treatments. Evidence provided here suggests that PPIs could be included in such design. As a proof of principle for such hypothesis, chemosensitization of tumors by concomitant treatment with proton pump inhibitors have been reported in spontaneously occurring animal tumors, mice xenografts and preclinical studies in osteosarcoma patients and with combinations with methotrexate, cisplatin, and adriamycin (22-25). Potential problems with long-term use of high doses of PPIs are still unknown since such studies are currently lacking.

Observed inhibition of  $H^+/K^+$ -ATPase proton pump caused a build-up of protons inside the cell, lowering the pH which correlates well with accepted mechanism of action for PPIs. Intracellular proton build-up consequently decreased breast cancer cell growth and survival.

Our findings suggest that responsiveness of MDA-MB-468 cells is mediated by gastric type of  $H^+/K^+$ -ATPase proton pump. Such findings are novel since expression of that pump was not previously reported in breast cancers. Further studies will be performed to investigate expression of  $H^+/K^+$ -ATPase proton pump by

immunohistochemistry using histological specimens of various breast cancers in order to correlate such expression with clinical features of various cancers with specific emphasis on TNBC.

Interestingly, certain isoforms of H<sup>+</sup>/K<sup>+</sup> ATPase (HKα1, HKα2, HKα3 and HKα4) have been described in non-gastric tissues such as kidney (26).



**Figure 3.** Sensitivity of MDA-MB-468 cells to esomeprazole is mediated by expression of gastric H<sup>+</sup>/K<sup>+</sup>-ATPase proton pump. Immunofluorescence analysis detected H<sup>+</sup>/K<sup>+</sup>-ATPase proton pump expression in MDA-MB-468 breast cancer cells (A) and not in MCF-10A control cells (B). These findings were confirmed by Western blots (B). Equal loading was determined by detection of a tubulin in both samples (C). Increased sensitivity to esomeprazole was also detected in MDA-MB-468 cells compared to MCF-10A control cells (D). These data were confirmed by treating both cell types with fixed concentration of esomeprazole (100 μM) (E).

Despite finding H<sup>+</sup>/K<sup>+</sup> ATPase in tissues other than stomach epithelium, to our knowledge, there are no reports of detecting that pump expression in TNBC or normal breast tissue.

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