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Original Articles

Proton pump inhibitors induce a caspase-independent antitumor effect against human multiple myeloma

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ABSTRACT

Multiple Myeloma (MM) is the second most common hematological malignancy and is responsive to a limited number of drugs. Unfortunately, to date, despite the introduction of novel drugs, no relevant increase in survival rates has been obtained. Proton pump inhibitors (PPIs) have been shown to have significant antitumor action as single agents as well as in combination with chemotherapy. This study investigates the potential anti-tumor effectiveness of two PPIs, Lansoprazole and Omeprazole, against human MM cells. We found that Lansoprazole exerts straightforward efficacy against myeloma cells, even at suboptimal concentrations (50 μM), while Omeprazole has limited cytotoxic action. The Lansoprazole anti-MM effect was mostly mediated by a caspase-independent apoptotic-like cytotoxicity, with only a secondary anti-proliferative action. This study provides clear evidence supporting the use of Lansoprazole in the strive against MM with an efficacy proven much higher than current therapeutical approaches and without reported side effects. It is however conceivable that, consistent with the results obtained in other human tumors, Lansoprazole may well be combined with existing anti-myeloma therapies with the aim to improve the low level of efficacy of the current strategies.

Introduction

Multiple Myeloma (MM) is the second most common hematological malignancy accounting for 10% of all hematological cancer diagnosed worldwide [1,2] and more than 60,000 deaths annually [3]. MM is the result of the clonal expansion of malignant plasma cells within the bone marrow, in turn leading to bone marrow depletion and paraneoplastic syndromes such as hyperglobulinemia, hyperviscosity, coagulation disorders and organ damage secondary to k chains deposition. Unfortunately, the current protocol of therapy against MM did not show to have dramatically changed the clinical response. In fact, despite the addition of new agents such as bortezomib, proteasome inhibitor (PI), thalidomide, lenalidomide and immunoModulatory drugs (IMiDs) to the chemotherapy protocols, this tumor remains poorly responsive and tends to become rapidly resistant to virtually all the tented therapies [4,5]. To date, death rates are still very high approaching 45%, and these rates have been stable since 2002 with no relevant improvement over the past 13 years [6]. Therefore, search for effective therapies against MM remains an unmet clinical need.

One common phenotype and possibly a hallmark of malignant tumors is the pH reversal (acidic extracellular pH and alkaline cytoplasmic pH) [7], due to a cascade of events beginning from the extracellular lactate accumulation (end product of anaerobic glycolysis, also known as "Warburg effect"), and leading to H^+ accumulation [8]. The acidic tumor microenvironment selects cells able to survive and proliferate in this hostile condition, in turn acquiring a series of malignant advantages including proliferation, invasion, metastasis and drug resistance. This is obtained by the upregulation of several classes of proton exchangers, among which V-ATPases play a pivotal role [9–11]. Lansoprazole (Lan) and Omeprazole (Ome) are specific proton pomp inhibitors (PPIs) which are currently used as anti-acid drugs for treatment of peptic diseases [12,13]. They are prodrugs which need an acidic environment in order to be activated [14]. Our preclinical investigations have shown that PPIs can be used as both chemosensitizers and direct antitumor agents as well [15–19]. Furthermore, several studies suggested that PPIs are able to reduce cancer acidity, thus countering the malignant behavior of cancer cells, including proliferation, drug resistance, invasiveness, and migration [20–23]. Previous investigations have shown the effectiveness of PPIs against different cancers, in both pre-clinical and clinical studies, with no evidence of side

Abbreviations: MM, multiple myeloma; PPIs, proton pump inhibitors; Lan, Lansoprazole; Ome, Omeprazole; PI, proteasome inhibitor; IMiDs, immunoModulatory drugs.

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effects [11,16,17,24–29]. Further studies have recently reported that, although PPIs belong to a family of generic drugs that worldwide are used regardless to some important differences in term of pharmacodynamics and bioavailability, actually they differ as far as the anti-neoplastic activity is concerned. In fact, our recent investigation has proven that among all PPIs, Lansoprazole was the agent showing the greatest antitumor potential even at doses lower than the other drugs of its family [19].

This study was aimed at investigating the effect of different proton pump inhibitors, such as Lansoprazole and Omeprazole (PPIs) against human multiple myeloma.

Materials and methods

Chemicals and reagents

PPIs were purchased as following: Omeprazole from Astra-Zeneca (Molndal, Sweden), whereas Lansoprazole by Sigma-Aldrich (Milan, Italy). The sodium salts were resuspended in physiologic solution or DMSO, respectively, at a concentration of 20 mM in the absence of direct light immediately before use. Trypan blue was bought from Alexis Biochemicals (Florence, Italy) and Annexin V-FITC apoptosis detection kit from Enzo Life Sciences (Lause, Switzerland); 4-nitrophenyl phosphate disodium salt hexahydrate tablets for proliferation assay from Sigma-Aldrich (Milan, Italy). The pan-caspase inhibitor z-VAD-fmk was from Calbiochem and used at 20 μmol/l.

Cell cultures

RPMI 8226 and U266 cell lines were obtained from American Type Culture Collection (ATCC Manassas, VA, USA) and cultured using modified conditions described by Matsuoka et al. and Nilsson et al. [30,31]. Briefly, both cell lines were maintained in RPMI-1640 media containing L-glutamine supplemented with 1% penicillin/streptomycin (Cambrex Bioscience, Walkersville, MD, USA) and 10% or 15% fetal bovine serum respectively by incubation at 37 °C and 5% CO_2. Experiments were performed in buffered medium at pH 6.5. The acid cell culture medium (pH 6.5) was obtained by the addition of 1 M HCl solution. The pH was estimated by the use of a pH 123 Microprocessor pH Meter (Hanna Instruments, Milan, Italy).

Cell death assay

The effects of PPIs treatment were evaluated on the two myeloma cell lines. Cells were plated at 4×10^5 cells/ml in 24-well plates in 0.5 ml per well of buffered medium at pH 6.5. After 48 hours, Lansoprazole or Omeprazole was added to the wells at increasing concentrations (50, 100, and 200 μM). 24 or 48 hours later, cells were collected by pooling cells from the medium, washed and resuspended in PBS with 0.05% trypan blue for 10 minutes at room temperature and analyzed by a dual-laser FACScalibur flow cytometer (BD Biosciences, Heidelberg, Germany) to determine the percentage of dead cells using CellQuestPro software. All experiments were run in triplicate wells and repeated at least twice.

Proliferation assay

The two myeloma cell lines were plated in 96-well plates at a concentration of 1×10^6/ml in buffered medium at pH 6.5. 48 hours later, Lansoprazole or Omeprazole was added to the wells. After 1 or 2 days of 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., Colmar Cedex, France). All experiments were run in triplicate wells and repeated at least twice.

Annexin V-PI apoptosis assay

Cells were plated at 3×10^5 cells/well in 24-well plates in 1 ml of buffered medium at pH 6.5. After 24 hours, Lansoprazole was added to the wells at increasing concentrations (50, 100, and 200 μM). 24 or 48 hours later, cells were collected for analysis. The involvement of caspases in Lansoprazole-mediated cell death was evaluated by using the pan-caspases inhibitor z-VAD-fmk. The caspases inhibitor was incubated with the cells 2 hours prior the addition of Lansoprazole. Cells were stained with Annexin V-FITC and propidium iodide for apoptosis detection (Enzo Life Sciences, Lausen, Switzerland) as reported in the manufacturer's instruction. Samples were then analyzed by a dual-laser FACScalibur cytometer (BD, Biosciences, Heidelberg, Germany) equipped with a 488 nm argon laser. Data were recorded and statistically analyzed by a Macintosh computer using CellQuestPro Software. The experiment was 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 < 0.05 were considered as statistically significant. Statistical analysis was performed with Sigmastat 3.0 software.

Results

PPIs show direct cytotoxicity against human myeloma cell lines

Our previous preclinical investigations have shown that PPIs can exert a direct anti-tumor activity both *in vitro* and *in vivo* against different tumor histotypes (melanoma, B-cell lines, osteosarcoma, glioblastoma) [11,17,18,32]. However, there was no evidence of the effect of PPIs against human multiple myeloma-derived cells, in either pre-clinical or clinical settings. In order to set up an experimental protocol we decided to use two PPIs for the treatment of these cells: (i) Omeprazole that has proven to exert a clear tumoricidal action against human B-cell malignancies [17]; (ii) Lansoprazole that was proven to be the most potent between the PPIs, in terms of cytotoxic action against tumor cells [19]. In fact, Lansoprazole exert its highest anti-tumor effect even at suboptimal concentration (50 μM) [19], also in combination with other drugs [16]. Taking into account these considerations, the purpose of these experiments was to evaluate whether treatment with the PPIs Omeprazole and Lansoprazole induces cytotoxicity in human myeloma cell lines *in vitro*. Their efficacy were tested at pH 6.5 cell culture condition, an acidic condition that at the same time approximated the pH values observed in tumors and allowed a full activation of PPIs [19].

To this aim, Omeprazole and Lansoprazole were added to the cultures 2 days after plating the cells, and viability was measured 24 and 48 hours later by FACS analysis of trypan blue-stained cells. These two sets of experiments showed first that Omeprazole was less cytotoxic than Lansoprazole. Moreover, the RPMI 8226 cells were more responsive than U266 (Fig. 1). Interestingly, Lansoprazole showed a clear tumoricidal action against both human myeloma cell lines and more remarkably against the RPMI 8226, while Omeprazole showed a full cytotoxic effect only against the RPMI 8226 cell line and just at the highest concentration (200 μM) after 48 hours of treatment (Fig. 1). More in detail, Lansoprazole induced 57% of cell death in experiments carried out with U266, while in RPMI 8226 the percentages of cell death peaked at 96% (Fig. 1). This first set of results showed that human multiple myeloma cells underwent cell death following treatment with PPIs and in particular with Lansoprazole.

PPIs did not significantly affect viability of human myeloma cell lines

To test whether the PPI-induced cytotoxicity correlated with an anti-proliferative effect, we evaluated whether treatment with the PPIs Omeprazole and Lansoprazole affected viability and proliferation of human myeloma cells. We then measured the proliferation of the two human myeloma cell lines in acidic culture medium in presence of both PPIs. The effect on myeloma cells proliferation was measured at 24 and 48 hours after the exposure to PPIs. The results showed that Omeprazole, tested against both RPMI 8226 and U266 myeloma cell lines, slightly inhibited cell proliferation and mostly at the highest concentration of 200 μM (Fig. 2), while Lansoprazole showed a not significant inhibitory effect beginning from the 50 μM concentration and peaking at the concentration of 200 μM (Fig. 2). Moreover, the anti-proliferative effect was more significant at 48 hours and against RPMI 8226 cells (Fig. 2). Specifically, the measured inhibitions at 48 hours were: 20% at 50 μM, 22% at 100 μM and 30% at 200 μM for the RPMI 8226 and 10% at 50 μM, 10% at 100 μM and 30% at 200 μM for the U266 (Fig. 2).