Combined Use of Zoledronic Acid Augments Ursolic Acid-Induced Apoptosis in Human Osteosarcoma Cells through Enhanced Oxidative Stress and Autophagy

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Introduction

Osteosarcoma is also known as osteogenic sarcoma which arises from osteoid tissue in the bone. This type of tumor most often localizes in the metaphysis of the adolescent long bones, which is characterized by a high propensity for metastasis, especially in lung. The outcome for this metastatic potential is frequently associated with high incidence of death in patients. The current treatments for osteosarcoma include surgery, radiation therapy, chemotherapy and other new forms of treatments, such as immunotherapy and targeted therapy. Unfortunately, there is a lack of effective cures for most patients suffering from advanced osteosarcoma. Recent progress has focused on the chemoprevention by natural products for their anti-growth activity against cancer cells and may exhibit less side effects compared to synthetic compounds. Ursolic Acid (UA) (3 -hydroxy-urs-12-en-28-oic acid) is a pentacyclic triterpenoid compound found in apple peels, and in the Ayurveda herb known as Holy Basil. UA is shown to trigger apoptosis, leading to anti-cancer, anti-invasion, anti-metastasis, anti-proliferation and anti-angiogenesis in an array of human cancer lines [1–6]. However, the studies on the effect of UA on human bone cancer cells are fairly limited. In this regard, we have just reported that UA also suppresses human osteosarcoma MG-63 cell growth through enhanced oxidative stress and ERK1/2-MAPK-assosiated apoptotic pathway. [7].

Zoledronic acid (ZOL), a third-generation nitrogen-containing bisphosphonate, is an inhibitor of osteoclast-mediated bone resorption and has demonstrated its efficacy in the treatment of bone metastases in cancer patients with breast, prostate, lung, and other solid tumors. Data in in vitro studies also support that ZOL inhibits osteosarcoma cell growth through activation of immune system, suppression of angiogenesis and apoptosis induction. Interestingly, the use of ZOL has also been shown to reverse drug resistance in osteosarcoma. Moreover, ZOL has the ability to reduce primary tumor growth, decrease lung metastases and prolong survival in animal models of osteosarcoma . In a four-patient cohort study, following initiation treatment of ZOL with high-grade osteosarcoma, the median progression-free survival was increased to 19 months, and median overall survival was increased to longer than 56 months. Given that ZOL exhibits great potential as an anti-cancer agent in bone cancer cells, we therefore sought to study the additive effect of the combination of ZOL and UA on human bone cancer cells.

Methods and Results

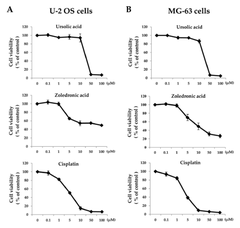


Fig 1. Zoledronic acid, ursolic acid, and cisplatin differentially affect osteosarcoma cell viability. MTT assays were performed with U-2 OS cells (A); or MG-63 cells (B) treated with either Zoledronic acid (ZOL), ursolic acid (UA), or cisplatin (CPT) at the indicated concentrations for 72 h. Three independent experiments were conducted.

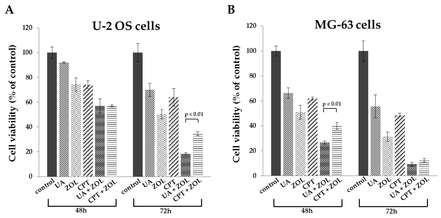


Fig 2. Zoledronic acid enhances ursolic acid-mediated inhibition of cell viability. MTT assays were performed with U-2 OS cells (A) or MG-63 cells; (B) treated with either ZOL (10 M), UA (20 M), CPT (5 M) or a combination of ZOL/UA, or ZOL/CPT at different time points. Three independent experiments were conducted.

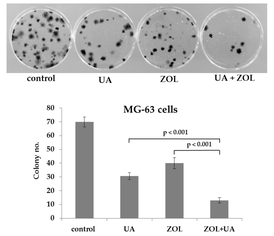


Fig 3. Zoledronic acid enhances ursolic acid-mediated reduction of colony formation. MG-63 cells were plated in colony formation assays after treatment with either ZOL (10 μM), UA (20 μM), or a combination of ZOL/UA for 6 h. Five hundred cells were plated per dish. All experiments were performed in triplicate, and the figure above shows a representative example.

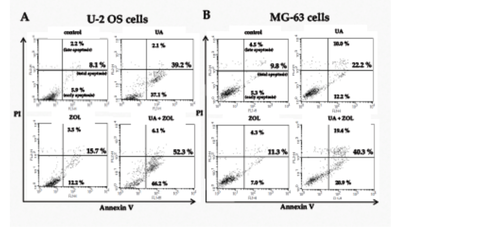


Fig 4. Zoledronic acid combines with ursolic acid to enhance apoptosis. U-2 OS cells (A); or MG-63 cells (B) were treated with either ZOL (10 M), UA (20 M) or a combination of ZOL and UA for 48 h. To detect apoptosis, the cells were stained with Annexin-V and propidium iodide (PI), and analyzed using flow cytometry. Numbers indicated the percentage of Annexin-V positive cells.

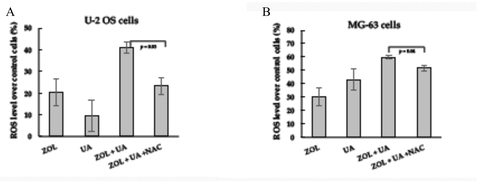


Fig 5. Zoledronic acid combines with ursolic acid to enhance reactive oxygen species (ROS) generation. U-2 OS cells (A) or MG-63 cells; (B) were treated with either ZOL (10 M), UA (20 M) or a combination of ZOL/UA in the absence or presence of N-acetyl-L-cysteine (NAC) (2 M) for 16 h. The cells were stained with hydroethidine (10 M) and ROS levels were analyzed by flow cytometry. Numbers indicated the induced levels of ROS in drug-treated cells compared to control cells. The results were calculated by a median of fluorescence intensity in each group.

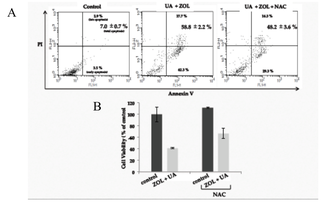


Fig 6. ROS is involved in the apoptosis effect of the UA/ZOL combination. (A) ROS inhibitor, N-acetyl-L-cysteine (NAC) decreases the apoptosis induced by the combination of ZOL and UA. U-2 OS cells were treated with ZOL (10 M) and UA (20 M) in the absence or presence of NAC (2 M) for 48 h. The treated cells were stained with Annexin-V and PI to analyze the percentage of apoptosis cells as indicated numbers by flow cytometry; and (B) NAC rescues the cell viability inhibition caused by the combination of ZOL and UA determined by MTT assays.

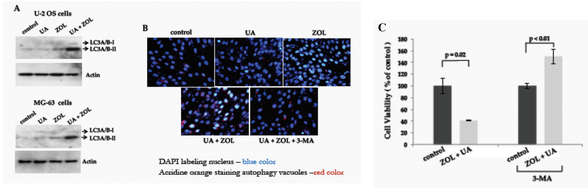
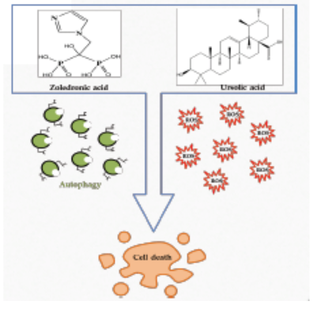


Fig 7. Autophagy is involved in the anti-proliferative activity of zoledronic acid/ursolic acid combination. (A) zoledronic acid combines with ursolic acid to induce the expression of LC3A/B-II. U-2 OS cells or MG-63 cells were treated with either ZOL (10 M), UA (20 M) or a combination of ZOL and UA for 48 h. Cells were harvested and the cell lysates were analyzed by Western blotting using indicated antibodies; (B) zoledronic acid combines with ursolic acid to induce acidic autophagic vacuoles. MG-63 cells were treated with either ZOL (10 M), UA (20 M) or a combination of ZOL and UA in the absence or present of 3-MA (2 M) for 36 h. The cells were stained with acridine orange (AO) to observe acidic (autophagic) vacuoles (red color), and 4',6-diamidino-2-phenylindole (DAPI) for labeling nucleus (blue color); and (C) autophagy inhibitor blocks the anti-proliferative effect caused by the UA/ZOL combination. U-2 OS cells were treated with ZOL (10 M) and UA (20 M) in the absence or presence of 3-MA (2 M) for 48 h. The treated cells were processed to MTT assays to measure cell viability. Cell viability of drug-treated cells was compared to their own control cells in the absence or presence of 3-MA.

Conclusion

Accumulating data have suggested that ROS signaling functions as the mediator of cell growth that either stimulate proliferation or induce cell death [8]. Previous studies have described that ZOL or UA treatment induce ROS-dependent apoptosis in different cancer cells [7,9,10]. Moreover, other groups have shown that ROS generation contributes to the significantly enhanced apoptosis by the combination of ZOL and ionizing radiation or panobinostat [11,12] Similarly, in this study, we found that either ZOL or UA triggered ROS elevation accompanied by apoptosis induction, and the UA/ZOL combination considerably enhanced these responses. Importantly, ROS scavenger NAC partially suppressed the apoptosis induced by the UA/ZOL combination, indicating that that ROS generation is associated with the combined effect of UA/ZOL in apoptosis induction in osteosarcoma cells.



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