INTRODUCTION:
We reported that third-generation bisphosphonates (BPs), YM175 and YM529 significantly inhibit the *in vitro* growth of murine osteosarcoma cell lines in a time- and dose-dependent manner by preventing prenylation of small GTPases and inducing apoptosis (51st ORS). And then, we reported that the combination of zoledronic acid (ZOL), another third-generation BP, with other anti-tumor drugs, could be more effective against murine osteosarcoma than the use of any of these drugs alone (52nd ORS). The aim of this study was to determine whether BPs can inhibit tumor growth or metastasis *in vivo*.

MATERIALS AND METHODS:
We used third-generation BPs, ZOL (Novartis). We used murine osteosarcoma cell line, LM8, which was established from the murine Dunn osteosarcoma cell line and has high metastatic potential to lung.

1. **Effect of ZOL on cell proliferation and cell cycle**

   The cell proliferation was determined by the trypan blue dye exclusion method and MTT assay. LM8 cells treated with 10 µM ZOL for 24 or 48 hours were analyzed for alterations in the cell cycle by staining with propidium iodide (PI) (Sigma Aldrich).

2. **Effect of ZOL on tumor cell adhesion, migration, and invasion**

   From the cell proliferation analysis, we found the non-toxic dose of ZOL for 24 hours. LM8 cells were incubated in collagen type I coated 96-well plates with 0.5, 1.0, and 2.0 µM of ZOL for 24 hours, and then we analyzed the adhered cells by MTT assay. Cell migration assay was performed using Bio-Coat cell migration chambers (Becton Dickinson). LM8 cells with or without ZOL were added to each insert (upper chamber), and the chemoattractant (10% FCS) was placed in each companion plate (lower chamber). After 24 hours incubation, the migrated cells were counted. Same as the migration experiments, cell invasion assay was performed using Matrigel coated invasion chambers.

3. **In vivo Effect of ZOL**

   LM8 cell line was stably transfected with the pGL3-control cector (Promega, Madison, WI) and with pSv2Neo (ATCC). Stable clones expressing luciferase (Luc) were isolated and the clone with the highest level of Luc expression was selected by using luciferin (Xenogen, Alameda, CA) and an *in vivo* imaging system (Xenogen).

   The mice were divided into three groups, namely, (i) untreated mice (n=6), (ii) mice treated with ZOL once a week (n=7), and (iii) mice treated with ZOL three times a week (n=7). 1 x 10^7 LM8Luc cells were injected into the subcutaneous tissue, and from the next day, we administered 80 µg/kg (body weight) of ZOL intraperitoneally. Before mice were anesthetized with Forane (Abbott), an aqueous solution of luciferin (150 mg/kg intraperitoneally) was injected 10 min prior to imaging. The animals were placed into the light-tight chamber of the CCD camera system (Xenogen) and the photons emitted from the luciferase-expressing cells within the animal were quantified for 5 min using the software program Living Image (Xenogen) as an overlay on Igor (WaveMetrics, Seattle, WA). We measured the tumor size and also analyzed the primary tumor site and the lung metastasis by using the *in vivo* imaging system.

RESULTS:

1. **Effect of ZOL on cell proliferation and cell cycle**

   The growth of LM8 cells was inhibited by ZOL in a time and dose dependent manner. The IC_{50} values at the time of 48 hours exposure was 7.36 µM (Figure 1A). In the histogram, after exposure for 24 or 48 hours, ZOL decreased the LM8 cells in the G2/M phase and increased in the S phase between the G0/G1 and G2/M phases (Figure 1B).

2. **Assessment of the ability of ZOL to block the growth of LM8*{in vitro}* by using in vivo imaging system**

   There was correlation between tumor volume and detected photons, by using the in vivo imaging system. Therefore we evaluated the efficacy of ZOL by measuring the photon counts of the primary tumor sites. There was no significantly effect of ZOL between groups (i) and (ii) after 4 weeks treatment. But the growth of group (iii) was significantly lower than that in group (i). (Figure 2).

3. **Effect of ZOL on tumor cell adhesion, migration, and invasion**

   ZOL was significantly blocked the adhesion ability on collagen type I of LM8 cells (Figure 3A). And also, ZOL was significantly blocked the migration and invasion ability of LM8 cells (Figure 3B, C).

4. **Assessment of the ability of ZOL to block lung metastasis of LM8*{in vivo}* by using in vivo imaging system**

   By using in vivo imaging system, when the primary subcutaneous tumor site was hidden by a black sheet, we could evaluate the lung metastatic lesions. The growth of the lung metastatic lesions of group (ii) and (iii) was significantly lower than that in group (i) (Figure 4).

DISCUSSION:
In this present study, we found that ZOL strongly inhibited the LM8 osteosarcoma cell growth and induced S-phase cell cycle arrest *in vitro*. But there was no increase in sub-G1 fraction, indicating that ZOL doesn’t induce LM8 cells into apoptosis. Then, ZOL inhibited significantly the primary tumor growth *in vivo*. To summarize, these results indicate that ZOL may inhibit LM8 osteosarcoma growth *in vivo* via the pathway of non-apoptotic cell death.

Furthermore, non-toxic dose of ZOL inhibited significantly the ability of adhesion, migration, and invasion. These are the principal factors when the malignant tumor comes into distant metastasis. In the result, ZOL also inhibited significantly the lung metastasis in both of the ZOL treated groups in *in vivo*. Therefore, low concentration of ZOL may inhibit tumor distant metastasis.

In conclusion, we confirm the direct anti-tumor effect of ZOL *both in vitro* and *in vivo* (anti-proliferative and anti-metastatic effect) against osteosarcoma cells. These results indicate that the third-generation BPs, ZOL, could offer benefits to osteosarcoma patients.