Accordingly, ZOL inhibits the prenylation of small G-proteins, such as Ras and RhoA, reduces the signals, and prevents the growth, adhesion, and migration of cancer cells.
70 ZOL has high affinity for mineralized bone, with rapid localization into the bone, resulting in therapeutically effective local concentrations for cancer cells.

Apart from breast and prostate cancers, there is little or no information on the effects of ZOL in HCC at a molecular level, although it has already been used clinically for the treatment of bone metastasis in HCC. In this study, we examined the
75 effects of ZOL in hepatoma cell lines *in vitro*.

Materials and Methods

Cell Culture. Human hepatoma cell lines Huh7 and HepG2 were used in this study. The cells were grown in William's Medium (Gibco BRL, Grand Island, NY)
80 supplemented with 10% fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), glutamax, and penicillin/streptomycin. Zoledronic acid was kindly provided by Novartis Pharmaceutical Company (Basel, Switzerland) . Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere. Then, adherent cells were incubated with the
85 indicated concentrations of ZOL for the indicated time periods.

Cell Proliferation. For the proliferation assay, we used the concentration of FBS (10%) to allow sufficient viability of Huh7 and HepG2 cells. Just after trypsinization, the cells were seeded at a concentration of $1x10^4$ cells per well in a 96-well plate and
90 incubated with ZOL for 24 hours. Cell viability was measured 48 hours later for Huh7

This article is protected by copyright. All rights reserved.

and HepG2 cells using the Cell Quanti-Blue Cell Viability Assay Kit (Bio Assay Systems, USA).

Cell migration. Cell migration was assembled according to the method of scratch assay.[19] Subconfluent growing cells were resuspended in type I collagen-coated 6-well plate. The plate was incubated for approximately 6 hours at 37°C, allowing cells to adhere, spread and create a confluent monolayer on the substrate. Then, the cell monolayer was scraped in a straight line to create a "scratch" with a p200 pipette tip. The debris was removed and the edge of scratch was smoothed by washing the cells once with 1 ml of phosphate buffered saline (PBS) , then with a medium containing predetermined concentration of ZOL. The markings were pointed on the substrate near the scratch to obtain the same field, and the scratch was observed and recorded under a phase-contrast microscope. The dish was placed in a tissue culture incubator at 37°C for more 18 hours. The scratch was observed and recorded under a phase-contrast microscope again and compared under these two phases.

Separation of proteins. After incubation with ZOL at different concentration for 24 hours, the proteins were separated using the following procedure. HCC cells were washed with cold PBS, lysed in ice-cold RIPA buffer containing phosphatase and protease inhibitors and centrifuged at 15000 rpm for 15 minutes at 4°C. The supernatant was collected as the full cell fraction. The protein concentration was determined by the method of Bradford using the Bio-Rad protein assay (Hercules, CA).

This article is protected by copyright. All rights reserved.

Accepted Article

115 **Immunoblotting Analysis.** The immunoprecipitated proteins were resuspended in loading buffer, boiled for 5 min at 99°C and further subjected to electrophoresis on SDS polyacrylamide gel. Equal amounts of protein extracts (50 μg) were subjected to PAGE under denaturing conditions. Proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membrane. Membranes were immunoblotted with 120 primary antibodies overnight at 4°C with pan-Ras and RhoA polyclonal antibodies (dilution, 1:1000, from Cell Signaling Technology, Beverly, MA), Erk1/2 and phospho-Erk1/2 (dilution, 1:1000, from Cell Signaling Technology), or Bak, Bax, Bcl-XL, MCL-1 monoclonal antibodies (dilution, 1:1000, from Cell Signaling Technology), followed by incubation with the appropriate mouse or rabbit derived 125 secondary antibody at room temperature for 1 hour and developed with ECL solution (Amersham Pharmacia Biotech, Piscataway, NJ) and exposed to Kodak X- OMAT films. Blots were rehybridized with β -actin polyclonal antibody (dilution, 1: 5000, Sigma-Aldrich Co., St. Louis, MO) to control protein loading.

130 **Immunohistochemical Analysis.** Immunohistochemistry was performed on Huh7 and HepG2 cells cultured on the slide glass in a 6-well plate. After incubation, the cells were washed in PBS, fixed with 3% paraformaldehyde/ 2% sucrose/ PBS for 10 minutes at room temperature, and permeablized with 0.5% Triton X. The fixed cells were incubated for 10 minutes at room temperature in PBS with 3% bovine serum 135 albumin (BSA) to avoid non-specific binding. The detection of Ras and RhoA was carried out by incubation of the cells with polyclonal primary antibodies against Ras

This article is protected by copyright. All rights reserved.

and RhoA for 1 hour at room temperature. After washing, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated mouse derived secondary antibody for 1 hour at room temperature. The cell membrane was leveled with anti-EpCAM polyclonal antibody (dilution, 1: 1000, Abcam, Cambridge, MA). To observe the formation of stress fibers, we also performed immunohistochemistry with β-actin polyclonal antibody as described above.

Flow Cytometric Analysis. Hepatoma cells were incubated with 1, 10, or 100 μM of ZOL for 48 hours. For the cell cycle assay, they were harvested and subjected to centrifugation, and $1x10^6$ cells/ml were incubated in the fluorochrome solution. DNA content was determined by flow cytometry and the cell cycle phase distribution was analyzed with BrdU flow kit (BD PharMingen, Franklin Lakes, NJ). Respective to their phase-specific DNA-content, the cells were divided into G0/G1, S, and G2/M phases.

TUNEL Assay analysis. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method was performed to label 3′-end of fragmented DNA of apoptotic Huh7 and HepG2 cells. The apoptotic cells were stained by the TUNEL method, using an *in situ* cell death detection kit (Roche Diagnostics, Basel, Switzerland), according to the instructions provided by the manufacturer. The FITC-labeled TUNEL-positive cells were imaged under a fluorescent microscopy at 488 nm excitation and 530 nm emission wavelengths.

This article is protected by copyright. All rights reserved.

160 **Caspase-3 Activity Assay.** Caspase-3 inactivates ICAD (inhibitor of caspase-activated deoxyrinonuclease) and indirectly activates CAD (caspase-activated deoxyrinonuclease), and it is related to chromatin fragmentation for nucleosome unit. We examined caspase-3 activity in ZOL-treated cells using Apopcyto Caspase-3 fluorometric Assay kit (Medical & Biological Laboratories, Nagoya, Japan).

165

Statistical Analysis. Results are expressed as mean±SEM. Differences between experimental groups were analyzed by the *t*-test. Differences were considered significant when $P<0.05$.

170 **Results**

ZOL inhibits proliferation of hepatoma cells. The effects of various concentrations of ZOL on the viability of Huh7 and HepG2 cells were studied after 48-hour incubation using the cell viability assay. ZOL reduced the viability of both Huh7 and HepG2 cells in a dose-dependent manner (Figure 1).

175 Either in the following analysis, similar changes were noted in HepG2 cells (data not shown).

ZOL inhibits hepatoma cell migration. Next, we analyzed the effects of ZOL on the migration of hepatoma cells using the scratch assay. The width of the line scraped on

180 the monolayer of Huh7 and HepG2 was measured under various concentrations of ZOL. Although ZOL had no effects on cell viability at the low concentrations (<10

This article is protected by copyright. All rights reserved.

μM), the cell migration into the scraped line was suppressed at concentrations ≥10 μM. (Figure 2)

185 **ZOL inhibits cell proliferation and migration by preventing cytosol-to-cell membrane translocation of small G-proteins.** As mentioned above, it is known that ZOL inhibits farnesyl pyrophosphate synthase, a key enzyme in the mevalonate pathway. First, we examined the effects of ZOL on the expression of RAS and RhoA, which are proteins located downstream of the mevalonate pathway. After incubation 190 with ZOL at different concentrations for 24 hours, proteins were extracted from the whole cell lysate and subjected to Immunoblotting. The latter showed similar expression patterns for small G-protein in the control cells and cells treated with ZOL (data not shown).

Prenylation is the addition of hydrophobic molecules to facilitate the 195 attachment of protein to the cell membranes. Prenylation of small G-proteins, such as Ras and RhoA, is considered an important process in protein-membrane interaction, which subsequently triggers cell proliferation and cell migration[20-23]. Therefore, we examined the effect of ZOL on the translocation of Ras and RhoA from the cytosol to the cell membrane of Huh7 and HepG2 cells. After 12-hour cell incubation, Ras was 200 observed in both the cytosol and cell membrane in the control and 10μM ZOL groups, but only in the cytosol of 100 μM ZOL-treated cells. Similarly, RhoA was observed in both the cytosol and membrane under control conditions, but only in the cytosol in 10 μM ZOL-treated cells (Figure 3). These findings suggest that ZOL inhibited

This article is protected by copyright. All rights reserved.

prenylation of these small G-proteins. Thus, inhibition of prenylation of RhoA altered
205 the formation of stress fibers and eventually changed the cell morphology (Figure 3).

In order to prove that Ras inhibition by ZOL was surely suppressed the cell viability, both cancer cells with Kras wild type and Kras mutant were treated with ZOL and then those cell viability was examined. Expectedly, the effectiveness of ZOL against Kras mutant cells was not so good as that against Kras wild cells.
210 (supplemental figure 1)

ZOL inhibits Erk1/2 phosphorylation , pro-apoptotic protein **overexpression, and promotes apoptosis of hepatoma cells.** In the next series of experiments, we examined the effects of inhibition of G-protein prenylation. To this end, we examined
215 MAPK pathway and apoptosis of hepatoma cells since they are associated with cell survival and cell death. ZOL inhibited Erk1/2 phosphorylation, which is phosphorylated downstream of Ras (Figure 4a). This result suggests that ZOL causes down-regulation of cell proliferation signals through the prevention of small G-protein prenylation. In another experiment, ZOL dose-dependently up-regulated Bak and Bax
220 in hepatoma cells (Figure 4a), but had no effect on the expression of Mcl-1 and Bcl-xL. The intensity is as below. Bak (control:1.2±0.2, 1μM:3.3±0.2, 10μM:4.5±0.7, 100μM:5.2±0.7), Bax (control:3.5±0.5, 1μM:3.8±0.6, 10μM:4.0±0.7, 100μM:4.6±0.7), Bcl-XL(control:7.1±0.9, 1μM:7.1±1.0, 10μM:7.2±1.3, 100μM:7.3±1.2), Mcl-1(control:1.6±0.5, 1μM:1.8±0.5, 10μM:1.9±0.5. 100μM:1.8±0.5),
225 β-actin(control:4.6±0.2, 1μM:4.6±0.2, 10μM 4.7±0.1, 100μM 4.7±0.1). (Average±SD, intensity; $\times 10^4$)

This article is protected by copyright. All rights reserved.

We also investigated the effect of ZOL on the cell cycle phase distribution of hepatoma cells after 48-hour treatment. The analysis demonstrated that approximately 74.0 % of Huh7 cells were in G0/G1, 14.4 % in G2/M, and 11.7 % in the S phase (Figure 4b). In contrast, ZOL dose-dependently increased the percentage of cells in the S phase.

Finally, we studied the effect of ZOL on apoptotic cell death. After 12-hour incubation, few TUNEL-positive cells were noted among cells incubated with low concentration of ZOL (<10 μM). However, the percentage of TUNEL-positive cells increased under high concentrations of ZOL (>10 μM) (Figure 5a). In other experiments, ZOL dose-dependently reduced caspase-3 activity in both Huh7 cells after 12-hour incubation (Figure 5b).

Discussion

Radiotherapy[24] and percutaneous cementoplasty[25] are currently being used for the treatment of bone metastasis from HCC, based on alleviation of pain and inhibition of tumor progression. However, the effects of both treatments are locoregional, therefore, it is difficult to select these treatment for multiple bone metastases. On the other hand, ZOL is a systemically administered drug, and considered the anti-tumor agent for multiple metastatic lesions.

Since the introduction of ZOL into clinical use for bone metastasis of solitary tumors in Japan, its benefits have been described in breast and prostate cancers, which tend to metastasize to bones. ZOL is also effective against HCC, and our group was among the first to report the clinical effects of ZOL against bone metastasis of HCC.[10]

This article is protected by copyright. All rights reserved.

250 Our retrospective cohort study investigated the efficacy of ZOL against bone metastases and reported that ZOL delayed both pain progression and radiographic progression of bone metastases, probably through prevention of migration of HCC into surrounding tissues. Accordingly, the present study analyzed the mechanism of the anti-tumor effect of ZOL on HCC study.

255 In the present study, we first analyzed the effects of ZOL on hepatoma cells. After 48-hour incubation at 37°C, ZOL dose-dependently reduced cell viability. Further analysis showed that ZOL has potent anti-invasive properties, preventing the migration of these cells. These findings suggested that ZOL modulates both cell growth and cell migration. Analysis of the anti-proliferative and anti-invasive effects 260 of ZOL showed inhibition of farnesyl pyrophosphate synthase, a key enzyme in the mevalonate pathway, with subsequent inhibition of prenylation of small G-proteins.[17, 18] Moreover, as a supplemental data, we confirmed that ZOL was not effective against Kras mutant cells, in which Kras has been activated spontaneously.

Next, we used immunohistochemistry to determine the effects of ZOL on the 265 translocation of small G- proteins cell from the cytoplasm to membrane and the effect of the latter on cell proliferation and migration. The results showed dyslocation of small G-protein after treatment with ZOL. These findings indicate the importance of small G-protein translocation in the activation of MAPK pathway and that ZOL can prevent such translocation. In this regard, Danoyelle *et al.*[26] reported similar results in 270 MDA-MB-231 cells.

The anti-proliferative effect of ZOL correlated also with changes in cell cycle distribution as indicated by the accumulation and arrest of tumor cells in the S-phase.

This article is protected by copyright. All rights reserved.

This observation is in agreement with the results of previous studies that examined the same effects on different types of tumor cells, which suggested arrest of ZOL-treated cells in the S-phase [27-31].

We also examined the role of ZOL on apoptosis. The Bcl-2 family proteins regulate the release of cytochrome c into the cytosol and control the activation of caspases and the apoptotic process. Bak and Bax are pro-apoptotic members of the Bcl-2 family, serve to release cytochrome c from the mitochondria, and initiate the caspase activation pathway for apoptosis. On the other hand, Mcl-1 and Bcl-xL antagonize the actions of pro-apoptotic Bcl-2 family members and inhibit apoptosis. The ratio of anti-apoptotic members to pro-apoptotic members might influence cell susceptibility to apoptosis. Our results showed exposure to ZOL activated the expression of pro-apoptotic proteins, decreased the anti-apoptotic proteins/pro-apoptotic proteins ratio, and promoted apoptosis, in hepatoma cells.

The present study on HCC and previous studies on breast cancer and prostate cancer[26, 32], highlighted the anti-tumor property of ZOL, which is based on inhibiting mevalonate pathway. This effect could be elicited not only on metastatic lesions but also on primary tumors, and should improve prognosis. Aft et al[33] reported recently better prognosis of breast cancer patients treated with ZOL compared to those without. Similarly, we reported previously that treatment with ZOL tends to improve prognosis of HCC patients with bone metastasis compared with non-ZOL based treatment.[10] These clinical outcomes support our finding that ZOL affects both metastatic bone tumor and primary tumor. These data prompt us to apply the combination therapies,

Accepted Article

This article is protected by copyright. All rights reserved.

such as ZOL plus radiotherapy or ZOL plus cementplasty, for systemic HCC metastases.

The present study has certain limitations. Although we confirmed the anti-tumor effect of ZOL, this effect was not tested using tumor tissue but tumor cell lines. It is uncertain whether the same anti-tumor effect and mechanism of action would be observed *in vivo*. Furthermore, there are data available on blood ZOL concentrations that are achieved during treatment of cancer patients. Although ZOL is usually taken up by osseous tissue and the remainder is excreted by the kidney within few hours, ZOL optimal dose need to be determined for each type of cancer, for both primary cancer and metastasis. Nevertheless, our data highlight the promising potential of ZOL in the treatment of bone metastasis from HCC.

Conclusion

Our *in vitro* study demonstrated that ZOL prevented cell growth and migration based on direct antitumor effect in HCC cell lines. The clinical use of ZOL could not only improve local control of bone metastasis but also prevent growth of primary lesion and new metastatic lesions of HCC. Further studies, including improvement of ZOL drug delivery or its use in combination with other chemotherapeutic agents, might help improve the prognosis of HCC patients with bone metastasis.

Conflict of interest

All authors declare no conflict of interest.

Accepted Article

This article is protected by copyright. All rights reserved.

Acknowledgement

Zoledronic acid was provided by Novartis Pharmaceutical Company (Basel,

320 Switzerland) for this study. Analysis of data of this study was carried out in cooperation with the analysis center of Life Science, Natural Science Center for Basic Research and Development, Hiroshima University.

Accepted Article

This article is protected by copyright. All rights reserved.

325 **References**

1 Nakano M, Ando E, Sato M *et al*: Recent progress in the management of hepatocellular carcinoma detected during a surveillance program in Japan. *Hepatol Res* 40:989-996, 2010.

2 Arii S, Sata M, Kudo M, *et al*: Management of hepatocellular carcinoma: Report 330 of Consensus Meeting in the 45th Annual Meeting of the Japan Society of Hepatology (2009). *Hepatol Res* 40: 667-685, 2010.

3 Okita K: Management of hepatocellular carcinoma in Japan. *J Gastroenterol* 41: 100-106, 2006.

4 Uka K, Aikata H, Takaki *et al*. Pretreatment predictor of response, time to 335 progression, and survival to intraarterial 5-fluorouracil/interferon combination therapy in patients with advanced hepatocellular carcinoma. *J Gastroenterol* 2007; 42:845-53.

5 Kamada K, Kitamoto M, Aikata H *et al*. Combination of transcatheter arterial chemoembolozation using cisplatin-lipiodol suspension and percutaneous ethanol 340 injection for treatment of advanced small hepatocellular carcinoma. *Am J Surg* 2002; 184: 284-90.

6 Rossi S, Di Stasi M, Buscarini E *et al*. Percutaneous RF interstitial thermal ablation in the treatment of hepatic cancer. *ALR Am J Roentgenol* 1996; 167:759-68.

345 7 Uka K, Aikata H, Takaki S *et al*. Clinical features and prognosis of patients with extrahepatic metastases from hepatocellular carcinoma. *World J Gastroenterol* 2007; 13:414-20.

This article is protected by copyright. All rights reserved.

8 Natsuizaka M, Omura T, Akaike T *et al.* Clinical features of hepatocellular carcinoma with extrahepatic metastases. *J Gastroenterol Hepatol* 2005; 20:1782-7.

350

9 Katyal S, Oliver JH III, Peterson MS, Ferris JV, Carr BS, Baron RL. Extrahepatic metastases from hepatocellular carcinoma. *Radiology* 2000; 216:698-703.

10 Katamura Y, Aikata H, Cyayama K *et al.* Zoledronic acid delays disease progression of bone metastases from hepatocellular carcinoma. *Hepatol Res* 2010; 40: 1195–1203, 2010.

355

11 Boissier S, Magnetto S, Frappart L *et al.* Bisphosphonates inhibit prostate and breast carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrices. *Cancer Res* 1997; 57: 3890-4.

12 Boissier S, Ferreras M, Peyruchaud O *et al.* Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. *Cancer Res* 2000; 60: 2949-54.

360

13 Fromigue O, Lagneaux L, Body JJ. Bisphosphonates induce breast cancer cell death in vitro. *J Bone Miner Res* 2000; 15: 2211-21.

14 Senaratne SG, Pirianov G, Mansi JL *et al.* Bisphosphonates induce apoptosis in human breast cancer cell lines. *Br J Cancer* 2000; 82: 1459-68.

365

15 Hiraga T, Williams PJ, Mundy GR *et al.* The bisphosphonate ibandronate promotes apoptosis in MDA-MB-231 human breast cancer cells in bone metastases. *Cancer Res* 2001; 61: 4418-24.

This article is protected by copyright. All rights reserved.

16 Jagdev SP, Coleman RE, Shipman CM *et al.* The bisphosphonate, zoledronic acid, induces apoptosis of breast cancer cells: evidence for synergy with paclitaxel. *Br J Cancer* 2001; 84: 1126-34.

17 Hosfield DJ, Zhang Y, Dougan DR et al. Structural basis for bisphosphonate-mediated inhibition of isoprenoid biosynthesis. *J Biol Chem.* 5: 8526-8529, 2004.

18 Dunford JE, Thompson K, Coxon FP *et al.* Structure-activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonates. *J Pharmacol Exp Ther.* 296: 235-242, 2001.

19 Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc.* 2: 329-33, 2007.

20 Santini D, Caraglia M, Vincenzi B *et al*: Mechanisms of disease: Preclinical reports of antineoplastic synergistic action of bisphosphonates. *Nat Clin Pract Oncol.* 3: 325-338, 2006.

21 Yoshioka K, Matsumura F, Akedo H *et al*: Small GTP-binding protein Rho stimulates the actomyosin system, leading to invasion of tumor cells. *J Biol Chem* 273: 5146-5154, 1998.

22 Kuroda J, Kimura S, Segawa H *et al.* The third-generation bisphosphonate zoledronate synergistically augments the anti-Ph+ leukemia activity of imatinib mesylate. *Blood* 15: 2229-35, 2003.

This article is protected by copyright. All rights reserved.

23 Kimura S, Kuroda J, Segawa H *et al*. Antiproliferative efficacy of the third-generation bisphosphonate, zoledronic acid, combined with other anticancer drugs in leukemic cell lines. *Int J Haematol*. 79:37-43, 2004.

24 Seong J, Koom WS, Park HC *et al*: Radiotherapy for painful bone metastases from hepatocellular carcinoma. *Liver Int* 25: 261-265. 2005.

25 Kodama H, Aikata H, Cyayama K *et al*: Efficacy of percutaneous cementoplasty for bone metastasis from hepatocellular carcinoma. *Oncology* 72:285-292, 2007.

26 Danoyelle C, Hong L, Soria C *et al*: New insights into the actions of bisphosphonate zoledronic acid in breast cancer cells by dual RhoA-dependent and –independent effects. *Br J Cancer* 88:1631-1640. 2003.

27 Coleman RE. Skeletal complications of malignancy. *Cancer* 1997;80 (Suppl. 8):1588-94.

28 Bloomfield DJ. Should bisphosphonates be part of the standard therapy of patients with multiple myeloma or bone metastases from other cancers? An evidence-based review. *J Clin Oncol* 1998;16:1218-25.

29 Rosen LS, Gordon D, Tchekmedyian S, Yanagihara R, Hirsh V, Krzakowski M, et al. Zoledronic acid versus placebo in the treatment of skeletal metastases in patients with lung cancer and other solid tumors: a phase III, double-blind, randomized trial-the Zoledronic Acid Lung Cancer and Other Solid Tumors Study Group. *J Clin Oncol* 2003;21:3150-7.

30 Caraglia M, D'Alessandro AM, Marra M, Giuberti G, Vitale G, Viscomi C, et al. The farnesyl transferase inhibitor R115777 (Zarnestra) synergistically enhances

This article is protected by copyright. All rights reserved.

growth inhibition and apoptosis induced on epidermoid cancer cells by zoledronic acid (Zometa) and pamidronate. *Oncogene* 2004;23:6900-13.

415 31 Yeh KY, McAdam AJ, Pulaski BA, Shastri N, Frelinger JG, Lord EM. IL-3 enhances both presentation of exogenous particulate antigen in association with class I major histocompatibility antigen and generation of primary tumor-specific cytolytic T lymphocytes. *J Immunol* 1998;160:5773-80.

32 Neville-Webbe HL, Rostami-Hogjegan A, Holen I *et al*: Sequence- and

420 schedule-dependent enhancement of zoledronic acid induced apoptosis by doxorubicin in breast and prostate cancer cells. *Int J Cancer* 20:364-371, 2005.

33 Aft RL, Naughton M, Weilbaecher K *et al*. Effect of (Neo) adjuvant zoledronic acid on disease-free and overall survival in clinical stage II/III breast cancer. *Br J Cancer* 107:7-11, 2012.

425

Accepted Article

This article is protected by copyright. All rights reserved.

Figure legends

Figure 1. Zoledronic acid inhibits hepatoma cell growth in a dose-dependent manner. Huh7 and HepG2 cells were seeded at 1×10^4 per well and the cell number was counted after 48 hours in the presence or absence of ZOL. Cell viability at each concentration of ZOL is indicated as percentage compared to that of hepatoma cells without ZOL. Data are mean ± SEM of 6 experiments.

Figure 2. Effect of increasing concentrations of ZOL on hepatoma cell migration through the scratched line. *Left:* The cell monolayer was scraped in a straight line using a p200 pipette tip, removed the debris, and added the indicated concentration of ZOL to the dishes. After 18-hour incubation at 37°C, we compared the width of the scratched line before and after incubation. *Right:* The mean and standard deviation of the width of the scratch line was calculated for each concentration of ZOL in 3 experiments. Similar changes were noted in HepG2 cells (data not shown).

Figure 3. Translocalization of Ras and RhoA under ZOL treatment. Huh7 cells were treated with or without ZOL for 12 hours. *Top:* The location of Ras and RhoA was analyzed by confocal microscopy. The cell membrane was labeled with anti-EpCAM antibody. *Bottom:* The stress fiber was labeled with anti-β-actin antibody. Abnormal stress fibers could be seen as green dots pointed with white arrow. Scale bar 20 μm. Similar changes were noted in HepG2 cells (data not shown).

This article is protected by copyright. All rights reserved.

Figure 4. Phosphorylation of Erk1/2. (a) *Left:* In MAPK cascade, downstream of
450 Ras, ZOL inhibited Erk1/2 phosphorylation. *Right:* Expression of anti-apoptotic
proteins/pro-apoptotic proteins. Note the dose-dependent up-regulation of Bak and
Bax in hepatoma cells. The numbers below indicate the signal intensity. Equal
amounts of protein were loaded. β-actin was used as a positive control. (b) *Top:*
Results of flow cytometric analysis of DNA content in Huh7 cells, in the absence or
455 presence of 1, 10, or 100 μM of ZOL for 48 hours. *Bottom:* Percentage of cells at each
cycle phase. Replicate experiments yielded similar results. Similar changes were
noted in HepG2 cells (data not shown).

Figure 5. ZOL induces apoptosis of hepatoma cells. (a)Confluent Huh7 cells were
460 incubated for 12 hours with ZOL at the indicated concentration. Images of
TUNEL-positive cells were captured by a fluorescence microscope (200×). (b) ZOL
dose-dependently reduced caspase-3 activity in Huh7 cells. Similar changes were
noted in HepG2 cells (data not shown).

465 **Supplemental method 1.**

Cell Proliferation. For the proliferation assay, we used the concentration of FBS
(10%) to allow sufficient viability of WiDr (Colon cancer cells with Kras wild type)
and DLD1 (Colon cancer cells with Kras mutant) cells. Just after trypsinization, the
cells were seeded at a concentration of $1x10^4$ cells per well in a 96-well plate and
470 incubated with ZOL for 24 hours. Cell viability was measured 48 hours later for WiDr

This article is protected by copyright. All rights reserved.

and DLD1 cells using the Cell Quanti-Blue Cell Viability Assay Kit (Bio Assay Systems, USA).

Supplemental method 2.

475 **Cell Proliferation.** For the proliferation assay, we used the concentration of FBS (10%) to allow sufficient viability of Huh7 and HepG2 cells. Just after trypsinization, the cells were seeded at a concentration of $1x10^4$ cells per well in a 96-well plate and incubated with ZOL for 24 hours. Cell viability was measured 48 hours later for Huh7 and HepG2 cells using the Cell Quanti-Blue Cell Viability Assay Kit (Bio Assay

480 Systems, USA).

Supplemental figure legend 1.

Zoledronic acid inhibits the cell growth of Kras wild type cells but not Kras

485 **mutant cells.** WiDr (Colon cancer cells with Kras wild type) and DLD1 (Colon cancer cells with Kras mutant) cells were seeded at $1x10^4$ per well and the cell number was counted after 12, 24, 36, and 48 hours in the presence or absence of ZOL. Cell viability at each concentration of ZOL is indicated as percentage compared to that of colon cancer cells without ZOL. Data are mean ± SEM of 6 experiments.

490

Supplemental figure legend 2.

Zoledronic acid inhibits hepatoma cell growth in a dose and time-dependent manner. Huh7 and HepG2 cells were seeded at $1x10^4$ per well and the cell number

This article is protected by copyright. All rights reserved.