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1           **A combination of a DNA-chimera siRNA against PLK-1 and zoledronic acid**  
2                           **suppresses the growth of malignant mesothelioma cells *in vitro***

3  
4   Eri Kawata<sup>a,b,1</sup>, Eishi Ashihara<sup>a,1\*</sup>, Yoko Nakagawa<sup>a</sup>, Takahiro Kiuchi<sup>a</sup>, Mai Ogura<sup>a</sup>,  
5   Hisayuku Yao<sup>a</sup>, Kazuki Sakai<sup>a</sup>, Ruriko Tanaka<sup>a</sup>, Rina Nagao<sup>a</sup>, Asumi Yokota<sup>a</sup>, Miki  
6   Takeuchi<sup>a</sup>, Shinya Kimura<sup>c</sup>, Hideyo Hirai<sup>a</sup>, and Taira Maekawa<sup>a</sup>

7  
8   <sup>a</sup>*Department of Transfusion Medicine & Cell Therapy, Kyoto University Hospital, 54*  
9   *Kawahara-cho Shogoin, Sakyo-ku, Kyoto 606-8507, Japan*

10   <sup>b</sup>*Division of Internal Medicine, Kyoto Second Red Cross Hospital, 355 Kamannza*  
11   *Marutamachi, Kamigyo-ku, Kyoto 602-8026, Japan*

12   <sup>c</sup>*Division of Hematology, Respiratory Medicine, and Oncology, Department of Internal*  
13   *Medicine, Faculty of Medicine, Saga University, 5-1-1 Nabeshima, Saga 849-8501,*  
14   *Japan*

15   <sup>1</sup>These authors contribute equal to this work.

16  
17   **\*Correspondence to:**

18       Eishi Ashihara,  
19       Department of Transfusion Medicine and Cell Therapy  
20       Kyoto University Hospital  
21       54 Kawahara-cho Shogoin  
22       Sakyo-ku, Kyoto 606-8507  
23       Japan  
24       Tel: +81-75-751-3630 / Fax: +81-75-751-4283  
25       e-mail: [ash0325@kuhp.kyoto-u.ac.jp](mailto:ash0325@kuhp.kyoto-u.ac.jp)

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1 **Abstract**

2           Although novel agents effective against malignant mesothelioma (MM) have  
3 been developed, the prognosis of patients with MM is still poor. We generated a  
4 DNA-chimeric siRNA against polo-like kinase-1 (PLK-1), which was more stable in  
5 human serum than the non-chimeric siRNA. The chimeric PLK-1 siRNA inhibited  
6 MM cell proliferation through the induction of apoptosis. Next, we investigated the  
7 effects of zoledronic acid (ZOL) on MM cells, and found that ZOL also induced  
8 apoptosis in MM cells. Furthermore, ZOL augmented the inhibitory effects of the  
9 PLK-1 siRNA. In conclusion, combining a PLK-1 siRNA with ZOL treatment is an  
10 attractive strategy against MM.

11

12 **Key Words:** PLK-1; RNA interference; DNA-chimeric siRNA; Bisphosphonate;  
13 Malignant mesothelioma

14

1 **1. Introduction**

2 Malignant mesothelioma (MM) is an aggressive tumor, which develops from  
3 the mesothelial surface of the pleural and peritoneal cavities. Asbestos is well-known  
4 as a carcinogen in MM and the incidence of MM is increasing worldwide [1].  
5 Although several surgical approaches have been proven to be effective [2,3], a  
6 combination of therapies including chemotherapeutic agents, radiation, and  
7 immunotherapy are required to fight the disease. However, in spite of the emergence of  
8 novel effective anticancer agents such as pemetrexed [4,5] and raltitrexed [6,7], the  
9 prognosis of patients with MM is still poor [8,9]. Therefore, the development of novel  
10 effective therapeutic strategies is essential to improve the prognosis of this disease.

11 RNA interference (RNAi) is a process involving sequence specific  
12 post-transcriptional gene silencing induced by double-stranded (ds) RNA. It is widely  
13 applied as a powerful tool in postgenomic research, and has been experimentally  
14 introduced into the field of cancer therapy. Synthetic, short interfering RNAs (siRNAs)  
15 for inducing RNAi are 19- to 21- nucleotide dsRNAs with two-nucleotide 3' overhangs  
16 at either end [10,11]. Unfortunately, siRNAs are degraded by endogenous nucleases  
17 when administered *in vivo*. Many techniques, including the use of DNA-chimeric  
18 siRNAs, have been developed to protect siRNAs from such degradation [10,12,13].

1 Previous investigations have revealed that their silencing activity is as powerful as that  
2 of non-chimeric siRNAs [12-14].

3 Polo-like kinase-1 (PLK-1) belongs to the PLK family of serine/threonine  
4 kinases and is highly conserved among eukaryotes. PLK-1 regulates cell division at  
5 several points during the mitotic phase of the cell cycle, including: mitotic entry  
6 through CDK1 activation, bipolar spindle formation, chromosome alignment,  
7 segregation of chromosomes, and cytokinesis [15,16]. Previous studies have reported  
8 that PLK-1 is overexpressed in cancerous tissues and that PLK-1 expression levels are  
9 tightly correlated with histological grades of tumors, clinical stages, and the patients'  
10 prognosis [17-20]. Thus, PLK-1 is considered to be a suitable target for cancer therapy,  
11 and several small molecular targeting agents have been used in clinical trials [21,22],  
12 while siRNAs against PLK-1 have been investigated in preclinical studies [19,20,23].

13 Bisphosphonates (BPs) are inhibitors of bone-resorption, and second- and  
14 third- generation BPs have been developed primarily to treat benign and malignant  
15 bone disease [24]. This class of drugs inhibits the proliferation of cancer cells by  
16 preventing the post-translational prenylation of small GTPases including the Ras  
17 family proteins [25]. We have demonstrated previously that third-generation BPs such  
18 as zoledronic acid (ZOL) and minodronic acid (YM529) have direct anti-tumor effects

1 against different cancer cells [26-30].

2 In the present study, we have investigated the effects of a DNA-chimeric  
3 PLK-1 siRNA and ZOL on MM cells *in vitro*. Our results show that these agents  
4 induce apoptosis and inhibit the proliferation of MM cells. In addition, we found that  
5 ZOL enhances the inhibitory effects of the PLK-1 siRNA.

6

## 7 **2. Materials and Methods**

### 8 *2.1. Cell lines, reagents, and animals*

9 The human MM cell lines H2452, H2052, H28, and 211H were cultured in  
10 RPMI1640 medium (Gibco, Tokyo, Japan) containing 10% heat-inactivated fetal calf  
11 serum (FCS; Invitrogen, Tokyo, Japan), *l*-glutamine (Gibco), and 1%  
12 penicillin-streptomycin (Gibco). The normal human dermal fibroblast (NHDF) cells  
13 were cultured in Dulbecco's Modified Eagle medium (DMEM; Gibco) containing 10%  
14 FCS, *l*-glutamine, and 1% penicillin-streptomycin. All cell lines were maintained at  
15 37°C in a fully humidified atmosphere of 5% CO<sub>2</sub> in air. All four MM cell lines were  
16 obtained from the American Type Culture Collection (Rockville, MD). Normal  
17 fibroblast NHDF cells were purchased from Kurabo (Osaka, Japan). LIC™  
18 Transfection Reagent (Hayashi Kasei, Tokyo, Japan) was used for transfection into

1 MM cells. ZOL (1-hydroxy-2-[1*H*-imidazole-1-yl]ethylidene-bisphosphonic acid) was  
2 obtained from Novalits Pharma AG (Basel, Switzerland).

3 We generated two types of siRNA against PLK-1 (GenBank accession number  
4 NM\_005030) using siDIRECT™ (alphaGEN Co, Ltd, Tokyo, Japan). One of the  
5 siRNAs contained of ribonucleotides and the other was a DNA-chimeric siRNA  
6 consisting partially of deoxyribonucleotides. The oligonucleotide sequences of the  
7 non-chimeric PLK-1 siRNA against PLK-1 were: sense strands, 5'-  
8 GCACCGAAACCGAGUUAUUCA-3' and that antisense strand,  
9 5'-AAUAAACUCGGUUUCGGUGCAG-3'. The sequences of the DNA-chimeric  
10 siRNA against PLK-1 were: sense strand, 5'-GCACCGAAACCGAggtattca-3', and  
11 antisense strand, 5'-aataacUCGGUUUCGGUGCAG-3'. This DNA-modified siRNA  
12 was constructed by substituting six ribonucleotides at the 5' end of the guide strand and  
13 the 3' end of the passenger strand with the cognate deoxyribonucleotides (designated in  
14 lower case). The oligonucleotide sequences for the chimeric siRNA controls were:  
15 sense strand, 5'-GUACCGCACGUCAttcgtatt-3', and antisense strand, 5'-  
16 tacgaaUGACGUGCGGUACGU-3'. The sequences for the non-chimeric control  
17 siRNA were: sense strand, 5'-GUACCGCACGUCAUUCGUAUU-3', and antisense  
18 strand, 5'-UACGAAUGACGUGCGGUACGU-3'. All siRNAs used were chemically

1 synthesized (Hokkaido System Science Co. Ltd, Hokkaido, Japan).

2

### 3 *2.2. Stability of the DNA-chimera siRNA in human serum*

4 We investigated the stability of the DNA-chimeric and non-chimeric siRNAs  
5 in human serum. Each siRNA was incubated in human serum (95%) at 37°C. Serum  
6 RNase was inactivated by adding SDS and proteinase K, and then digested samples  
7 were loaded onto 15% polyacrylamide gel, which was then stained using SYBR Gold  
8 (Invitrogen).

9

### 10 *2.3. Growth inhibitory effects of PLK-1 siRNA*

11 Cell proliferation was determined by the modified MTT assay using the Cell-  
12 Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan) as previously described [19;  
13 20]. Cells were seeded in a flat-bottomed 96-well plate (Becton Dickinson, Tokyo,  
14 Japan) at  $3 \times 10^3$  cells in 100 $\mu$ l of medium per well and incubated with serial dilutions  
15 of the DNA-chimeric siRNA for 72 hours. The mean of four samples was calculated.  
16 Half-maximal inhibition constants ( $IC_{50}$ s) were determined with the nonlinear  
17 regression program CalcuSyn (Biosoft, Cambridge, UK).

18



1    2.4. *Growth inhibitory effects of Zoledronic acid*

2           Cell proliferation was determined by the modified MTT assay using the Cell-  
3    Counting Kit-8 as mentioned above. Cells were seeded in a flat-bottomed 96-well plate  
4    (Becton Dickinson, Tokyo, Japan) at  $3 \times 10^3$  cells in 100 $\mu$ l of medium per well and  
5    incubated with serial dilutions of ZOL for 72 hours. The mean of four samples was  
6    calculated. Half-maximal inhibition constants ( $IC_{50}$ s) were determined with the  
7    nonlinear regression program CalcuSyn. We also evaluated the combined effects of  
8    concurrent PLK-1 siRNA and ZOL treatment on H2452 and H28 mesothelioma cell  
9    lines and the analyzed data is shown by the combination index (CI). CI is a method for  
10   quantifying drug cytotoxic synergism based on the mass-action law principle derived  
11   from enzyme kinetic models. This method was developed by Chou and Talalay [31,32]  
12   which has been widely used to evaluate interactions of antineoplastic agents [33-36].  
13   Cells were incubated for 72 hours with six concentrations (0.25, 0.5, 0.75, 1.0, 1.5, or  
14   2.0 times the  $IC_{50}$ ) of each agent or both in combination using the constant ratio design  
15   followed by the modified MTT assay. We calculated the combination indexes (CIs) as  
16   reported previously [33-36], and calculated the fraction affected (Fa) at each dilution  
17   (for example, Fa of 0.25 equals 75% viable cells). This method provides a  
18   quantification of the synergism ( $CI < 1$ ), additive effect ( $CI = 1$ ), and antagonism ( $CI >$

1 1) at different dose and effect levels [31]. Calculations of the CI were made under the  
2 assumption that the mechanisms of action of the evaluated drugs were not mutually  
3 exclusive.

4

#### 5 *2.5. Cell cycle and apoptosis analysis*

6 Cell cycle analysis using propidium iodide (PI) was performed as previously  
7 described [20]. Apoptosis induced by each siRNA treatment or ZOL treatment was  
8 determined using the Annexin-V-FITC Apoptosis Detection Kit I (BD Pharmingen,  
9 San Jose, CA) as recommended by the manufacturer. Cells were analyzed with FACS  
10 CANTO II using Diva software (BD Bioscience).

11

#### 12 *2.6. Western blotting analysis*

13 Following the transfection of cells with PLK-1 siRNA, or treatment with ZOL,  
14 as described above, the medium was aspirated and the cells were washed with ice-cold  
15 PBS (-). The cells were lysed with ice-cold RIPA buffer (50 mM Tris-HCl [pH 7.4],  
16 0.25 M NaCl, 5 mM EDTA, 20 mM NaF, 1% NP-40) with PMSF (1 mM) and protease  
17 inhibitor (10 µg/ml). The cells were then scraped off the plate, and the suspension of  
18 cells in lysis buffer was transferred to a centrifuge tube, which was placed on ice for 15

1 minutes with an occasional vortex to ensure complete lysis. The cell suspension was  
2 then cleared by centrifugation at 14,000g for 30 minutes at 4°C, and the supernatant  
3 (total cell lysis) was either used immediately or stored at -80°C. The protein  
4 concentration was determined using the DC Protein Assay (Bio-Rad Laboratories,  
5 Osaka, Japan).

6 Immunoblotting was performed as previously described [20]. The following  
7 primary antibodies (Abs) were used: rabbit polyclonal anti-PLK-1 Ab (Upstate  
8 Biotechnology Inc., Charlottesville, VA); rabbit polyclonal anti-caspase-3 Ab; rabbit  
9 polyclonal anti-cleaved caspase-3 Ab (Cell Signaling Technology, Danvers, MA);  
10 polyclonal anti-Rap1A Ab (Santa Cruz Biotechnology, Santa Cruz, CA); mouse  
11 monoclonal anti-RhoA Ab (Santa Cruz Biotechnology); mouse monoclonal anti-Ras  
12 Ab (BD Bioscience), and rabbit polyclonal anti-actin Ab (Sigma-Aldrich, Tokyo,  
13 Japan).

14

### 15 **3. Results**

#### 16 *3.1. Stability of the DNA-chimera siRNA in human serum*

17 An siRNA can be protected from RNase or nuclease cleavage by the partial  
18 substitution of ribonucleotides with deoxyribonucleotides at the 5' end of the guide

1 strand and the 3' end of the passenger strand. Therefore, we first designed the PLK-1  
2 siRNA using siDIRECT™ and then converted this siRNA into a DNA-chimeric siRNA.  
3 We incubated the DNA-chimeric, or non-chimeric siRNAs against PLK-1 in 95%  
4 human serum and investigated their degeneration. The non-chimeric siRNA  
5 degenerated in a time-dependent manner, while the DNA-chimeric siRNA did not  
6 degenerate for at least 120 minutes (Fig. 1A). This result shows that the chimeric  
7 siRNA is more stable in human serum than the non-chimeric siRNA.

8

### 9 *3.2. DNA-chimeric PLK-1 siRNA inhibited the growth of mesothelioma cells*

10 We examined the PLK-1 expression in four MM cell lines: H2452, H2042,  
11 H28, and 211H cell lines. All cell lines examined expressed a higher level of PLK-1  
12 than normal NHDF fibroblast cells (Fig. 1B). Next we confirmed the knockdown  
13 effects of both DNA-chimeric and non-chimeric PLK-1 siRNAs in MM cells. We  
14 transfected both types of siRNAs into H2452 MM cells, and both siRNAs effectively  
15 knocked down PLK-1 expression (Fig. 1C). Then we investigated the inhibitory effects  
16 of the DNA-chimeric PLK-1 siRNA on MM cells *in vitro*. Western blot analysis  
17 showed that the transfection of the DNA-chimeric PLK-1 siRNA suppressed PLK-1  
18 expression in H2452 mesothelioma cells in a dose-dependent manner, whereas the

1 nonsense chimeric siRNA (100 nM) did not (Fig. 2A). The IC<sub>50</sub> values for H2452 and  
2 H28 cells at 72 hours exposure were 1.6 nM and 38.7 nM, respectively. Out next step  
3 was to examine the growth inhibitory effects of the DNA-chimeric siRNA against  
4 PLK-1 on H2452 and H28 mesothelioma cells using a modified MTT assay. As shown  
5 in Fig. 2B, the chimeric PLK-1 siRNA inhibited cell growth in a dose-dependent  
6 manner, whereas no significant inhibitory effects were detected in normal NHDF cells  
7 (Fig. 2C).

### 9 *3.3. The mechanisms of cell death induced by PLK-1 depletion*

10 Next we investigated the mechanisms of cell death caused by PLK-1 siRNA  
11 transfection. Cell cycle analysis confirmed that PLK-1 siRNA treatment induced G2/M  
12 arrest as previously reported [19,20], and revealed an increase in the subG1 fraction 72  
13 hours after transfection with the DNA-chimeric PLK-1 siRNA (Fig. 3A, upper panel).  
14 Early apoptotic cells (Annexin-V+/PI- fraction), and late apoptotic cells and necrotic  
15 cells (Annexin-V+/PI+ fraction) also increased after DNA-chimeric PLK-1 siRNA  
16 transfection (Fig. 3A, lower panel). In addition, Western blotting analysis demonstrated  
17 an increase in cleaved caspase-3 activity following PLK-1 siRNA transfection (Fig.  
18 3B). Thus, transfection with a PLK-1 siRNA transfection resulted in the induction of

1 apoptosis in mesothelioma cells through the activation of caspase-3.

2

3 *3.4. ZOL inhibits the growth of mesothelioma cells and synergistically augments with*  
4 *the effects of the PLK-1 siRNA*

5 We examined the inhibitory effects of ZOL on H2452 and H28 mesothelioma  
6 cells using a modified MTT assay. ZOL inhibited cell growth in a dose-dependent  
7 manner, and the IC<sub>50</sub> values for H2452 and H28 cells at 72 hours exposures were 11.4  
8 μM and 58.1 μM, respectively (Fig. 4A). ZOL treatment increased the subG1 fractions  
9 (Fig. 4B) and the number of apoptotic cells (Fig. 4C) in a dose-dependent manner.  
10 Furthermore, we found that caspase-3 was cleaved by ZOL treatment (Fig. 5A).

11 Next we investigated the unprenylation of Rap1A, RhoA, and Ras proteins.  
12 MM cell lysates were analyzed by Western blotting using Abs against Ras and the  
13 unprenylated form of Rap1A and RhoA. ZOL treatment resulted in an increase in  
14 unprenylated Rap1A and RhoA in MM cells (Fig. 5B). The anti-Ras Ab recognizes  
15 both a slower migrating band, representing the unprenylated Ras, and a faster  
16 migrating band representing the prenylated Ras [37]. After ZOL treatment, there was  
17 an increase in the unprenylated form of Ras in MM cells which was accompanied by a  
18 reduction in the prenylated form (Fig. 5B). Taken together, the results indicate that

1 ZOL treatment induced apoptosis through the cleavage of caspase by blocking the  
2 prenylation of small GTP-binding proteins, which resulted in the inhibition of cell  
3 growth of MM cells.

4 We then investigated the combined effects of ZOL treatment with the PLK-1  
5 siRNA on H2452 and H28 MM cells. PLK-1 regulates RhoA in the mitotic phase  
6 [38,39]. The modified MTT assay with six concentrations (0.25, 0.5, 0.75, 1.0, 1.5, or  
7 2.0 times the  $IC_{50}$ ) of each agent or both in combination with the constant ratio was  
8 carried out. The values of the  $IC_{50}$  which were obtained from the experiments above  
9 were used. We calculated the CIs and the Fa values at each dilution using the CalcuSyn  
10 soft as reported previously [33-36]. Dose-effect and CI-Fa plots illustrating the effects  
11 of PLK-1 siRNA and ZOL combinations are shown in Figure 5C. As shown in the left  
12 panel of Figure 5C, the treatment of PLK-1 siRNA combined with ZOL produced more  
13 growth inhibition than the treatments of each agent alone that are shown in Figures 2B  
14 and 4A. The mathematically analyzed data of CI-Fa plots are shown in the right panel  
15 of Figure 5C. In H2452 cells, the CI values at Fa 0.5, and 0.8 were 0.809 and 0.974,  
16 respectively; and in H28 cells, the CI values at Fa 0.5, and 0.8 were 0.082 and 0.836,  
17 respectively. These observations indicate that exposure to ZOL and the DNA-chimeric  
18 PLK-1 siRNA produced a synergistic effect on H2452 and H28 MM cell lines. We also

1 investigated the alternation of the unprenylated RhoA expression in H2452 and H28  
2 cells by the treatment of PLK-1 siRNA and ZOL. The combined treatment of  
3 concurrent PLK-1 siRNA and ZOL did not alter the unprenylated RhoA expression  
4 compared to the treatment of ZOL alone (Supplementary Figure 1), suggesting that  
5 PLK-1 siRNA does not act on the prenylation of RhoA GTPase although PLK-1  
6 siRNA diminishes PLK-1 expression.

7

## 8 **Discussion**

9 Synthetic siRNAs form complexes with liposomes, after which, the  
10 siRNA/liposome complex binds to the cell membrane and enters the cytoplasm via  
11 endocytosis. The complex then escapes from the endosome and releases its siRNA to  
12 the RNAi machinery [11,40]. Although single-stranded (ss) nucleic acids are rapidly  
13 degraded in serum or inside cells, ds nucleic acids are more stable than their ss  
14 counterparts; however, ds nucleic acids, including siRNAs, are still degraded and must  
15 be protected from endogenous nucleases in the bloodstream. Degradation can be  
16 avoided by the use of a suitable delivery system or by the nuclease-resistant chemical  
17 modification of the siRNA [10]. One approach involves the modification of the  
18 2'-position of the ribose of the siRNA. Sugar modifications such as 2'-*O*-methylation,



1 2'-*O*-methoxyethylation, and 2'-fluoro-2'-deoxynucleoside modification can improve  
2 nuclease resistance [41,42]. Another approach involves the replacement of certain  
3 siRNA ribonucleotides with their deoxyribonucleotide counterparts.

4 We generated a DNA-chimeric siRNA, and substituted six basepairs from the  
5 5' end of the guide strand with their deoxyribonucleotide counterparts. This  
6 DNA-chimeric siRNA was more stable in human serum than the non-chimeric siRNA  
7 and showed resistance to endogenous nucleases. Moreover, the chimeric siRNA  
8 against PLK-1 decreased PLK-1 expression almost as effectively as the non-chimeric  
9 siRNA, and inhibited the proliferation of MM cells through the induction of apoptosis  
10 by cleaving caspase-3. These anti-neoplastic effects in the present study are consistent  
11 with our previous reports [19,20]. PLK-1 is overexpressed in MM cells compared to  
12 normal fibroblasts and, therefore, PLK-1 is a novel target that inhibits the proliferation  
13 of MM cells. Ui-Tei et al. [13] also demonstrated that DNA-chimeric siRNAs, in  
14 which the eight ribonucleotides from the 5' end of the guide strand were substituted  
15 with deoxyribonucleotides, effectively induced gene-silencing without exerting the  
16 off-target effects. Our findings, taken together with the Ui-Tei's report, demonstrate  
17 that DNA-chimeric siRNAs result in safe and more effective gene-silencing and that  
18 the DNA-chimeric siRNA is much suitable for an *in vivo* administration. Further

1 studies are warranted to evaluate the therapeutic potential of the DNA-chimeric siRNA  
2 *in vivo*.

3 ZOL, a third-generation BP, inhibits the activity of farnesyl diphosphate  
4 synthase in the mevalonate pathway, resulting in inhibition of the prenylation of small  
5 GTPases [24,25]. The prenylated small GTPases including Ras, Rap-1, and Rho  
6 proteins transduce signals downstream for cell functions such as cell proliferation,  
7 adhesion, and migration [43-45], while the inhibition of these small GTPase  
8 prenylation by BPs results in the suppression of cancer progression [26-30,46-48]. In  
9 the present study, we have revealed that ZOL also inhibits the proliferation of MM  
10 cells. Treatment of MM cells with ZOL inhibited the prenylation of Rap-1A, Ras, and  
11 RhoA proteins, and induced apoptosis by cleaving capsase-3. Other investigators have  
12 also reported that ZOL inhibits the proliferation of MM cells [49,50]. Interestingly, one  
13 report demonstrated that  $Ca^{2+}$  regulates the growth inhibitory effects of BPs on MM  
14 cells [49]. Calcification is a well-known feature in MM [51,52], and BPs accumulate  
15 rapidly in bone [24,25]. We have demonstrated previously that ZOL has antitumor  
16 effects against osteosarcoma [29,46]. These findings collectively suggest that  
17 malignancies with ossifying features, including MM and breast cancers [48], are  
18 suitable candidates for ZOL treatment.

1 PLK-1 regulates cell division at several points during the mitotic phase, and  
2 RhoA is also implicated in the regulation of cytokinesis. Prenylated Rho proteins are  
3 delivered to the plasma membrane and are concentrated at the cleavage furrow of the  
4 cell during the M phase of cell cycle [53,54]. PLK-1 regulates the local activation of  
5 RhoA to promote cytokinesis [38,39] and, therefore, we hypothesized that ZOL  
6 treatment would augment the cytotoxic efficacy of the PLK-1 siRNA in MM cells. The  
7 combined ZOL and PLK-1 siRNA treatment showed synergistic effects at both low (Fa  
8 0.5) and high (Fa 0.8) concentrations.

9 In conclusion, a DNA-chimeric PLK-1 siRNA inhibited cellular proliferation  
10 and induced apoptosis in MM cells, and a combination of PLK-1 siRNA and ZOL  
11 treatment revealed synergistic inhibitory effects on MM cells. The observation reported  
12 in the present study indicates that PLK-1 is a novel target for the treatment of MM and  
13 that the DNA-chimeric siRNA against PLK-1 combined with ZOL treatment would be  
14 an attractive strategy in the fight against this aggressive disease.

15

16

1    **Conflicts of interest**

2            All authors declare that they have no conflicts of interest.

3

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12

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13

1 **Figure Legends**

2

3 **Fig. 1.** DNA-chimeric siRNA against PLK-1 is more stable in human serum than a  
4 non-chimeric siRNA. (A) Each siRNA was incubated in human serum (95%) at 37°C.  
5 Serum RNase was inactivated by adding SDS and proteinase K, and then digested  
6 samples were loaded onto a 15% polyacrylamide gel. The gel was stained by SYBR  
7 Gold. a: non-chimeric PLK-1 siRNA, b: DNA-chimeric PLK-1 siRNA. (B) PLK-1  
8 expression in MM cells. Immunoblotting of whole cell lysates obtained from MM cell  
9 lines and normal NHDF human fibroblast cells. (C) Depletion of PLK-1 expression in  
10 H2452 MM cells in response to treatment with non-chimeric or DNA-chimeric PLK-1  
11 siRNA. We obtained whole cell lysates from H2452 MM cells 72 hours after the  
12 transfection of non-chimeric or DNA-chimeric PLK-1 siRNA (50 nM), and  
13 immunoblotting was performed as described in **Materials and Methods**.

14

15 **Fig. 2.** DNA-chimeric PLK-1 siRNA inhibits the proliferation of MM cells, but not  
16 NHDF normal fibroblast cells. (A) Expression of PLK-1 in H2452 MM cell lines.  
17 H2452 cells were incubated with serial dilutions of DNA-chimeric PLK-1 siRNA and  
18 LIC transfection reagent for 72 hours. Whole cell lysates were obtained and

1 immunoblotting was performed as described in **Materials and Methods**. (B) Cell  
2 proliferation was determined by the modified MTT assay as described in **Materials**  
3 **and Methods**. DNA-chimeric PLK-1 siRNA shows inhibitory growth effects on  
4 H2452 and H28 MM cells in a dose-dependent manner. (C) DNA-chimeric PLK-1  
5 siRNA does not inhibit the proliferation of normal NHDF fibroblast cells. Data  
6 represents the means  $\pm$  standard deviations (SD) of three independent experiments.  
7 Solid and dotted lines indicate chimeric PLK-1 siRNA and chimeric control siRNA,  
8 respectively.

9  
10 **Fig. 3.** DNA-chimeric PLK-1 siRNA treatment induces apoptosis in H2452 MM cells  
11 by activating caspase-3. (A: upper panels) Cell cycle analysis in H2454 MM cells  
12 using propidium iodide (PI) was performed after 72 hours of treatment with the  
13 chimeric control, or chimeric PLK-1 siRNAs, at the concentration indicated. Results  
14 are representative of three independent experiments. The numbers inside each  
15 histogram indicate the percentage of the subG1 fraction. (A: lower panels)  
16 Determination of apoptosis induced by each siRNA treatment. Results are  
17 representative of three independent experiments. The numbers inside each quadrant  
18 indicate the percentage of the cell population with the quadrant characteristic. (B)



1 Cleavage of caspase-3 by DNA-chimeric PLK-1 siRNA treatment. H2452 cells were  
2 incubated with serial dilutions of DNA-chimeric PLK-1 siRNA and LIC transfection  
3 reagent for 72 hours. Whole cell lysates were obtained and immunoblotting was  
4 performed as described in **Materials and Methods**. a: chimeric control siRNA, b:  
5 chimeric PLK-1 siRNA.

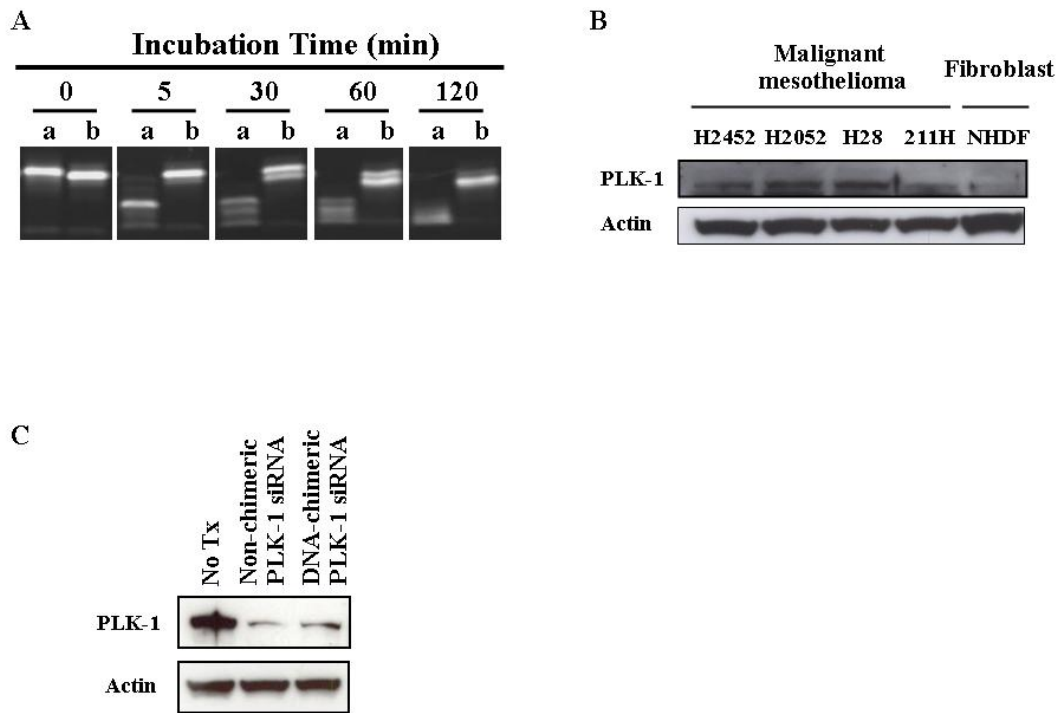
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7 **Fig. 4.** ZOL treatment inhibits the proliferation of MM cells. (A) Cell proliferation was  
8 determined by the modified MTT assay as described in **Materials and Methods**. ZOL  
9 treatment produced growth inhibitory effects in H2452 (solid line) and H28 (dotted  
10 line) MM cells in a dose-dependent manner. (B) Cell cycle analysis in H2452 MM  
11 cells using propidium iodide (PI) was performed after 72 hours treatment with ZOL at  
12 the concentration indicated. Results are representative of three independent  
13 experiments. The numbers inside each histogram indicate the percentage of the subG1  
14 fraction. (C) Determination of apoptosis induced by ZOL treatment at each  
15 concentration. Results are representative of three independent experiments. The  
16 numbers inside each quadrant indicate the percentage of the cell population with the  
17 quadrant characteristic.

18

1 **Fig. 5.** Induction of apoptosis in MM cells by ZOL treatment (A,B) and combined  
2 effects of chimeric PLK-1 siRNA with ZOL. (A) Cleavage of caspase-3 by ZOL  
3 treatment. Four MM cell lines were incubated with ZOL at 50  $\mu$ M for 72 hours. Whole  
4 cell lysates were obtained and immunoblotting was performed as described in  
5 **Materials and Methods.** (B) Evaluation of the inhibition of small GTPase protein  
6 prenylation by ZOL treatment (50  $\mu$ M). Immunoblotting and immunoprecipitation  
7 were performed as described in **Materials and Methods.** (C) Evaluation of the  
8 combined effects of DNA-chimeric PLK-1 siRNA and ZOL treatment on H2452 (filled  
9 circles) and H28 (filled triangles) mesothelioma cell lines. Cells were incubated for 72  
10 hours with six concentrations (0.25, 0.5, 0.75, 1.0, 1.5, or 2.0 times the  $IC_{50}$ ) of each  
11 agent or both in combination using the constant ratio design followed by the modified  
12 MTT assay as described in **Materials and Methods.** The  $IC_{50}$  values of PLK-1 siRNA  
13 for H2452 cells and H28 cells were 1.6 nM and 38.7 nM, respectively, and those of  
14 ZOL were 11.4  $\mu$ M and 58.1  $\mu$ M, respectively. Left panel: The killing curves of the  
15 concurrent administration of PLK-1 siRNA and ZOL. Right panel: Combination index  
16 (CI)-fraction affected (Fa) plots. Combination indexes were determined with the  
17 nonlinear regression program CalcuSyn.  
18

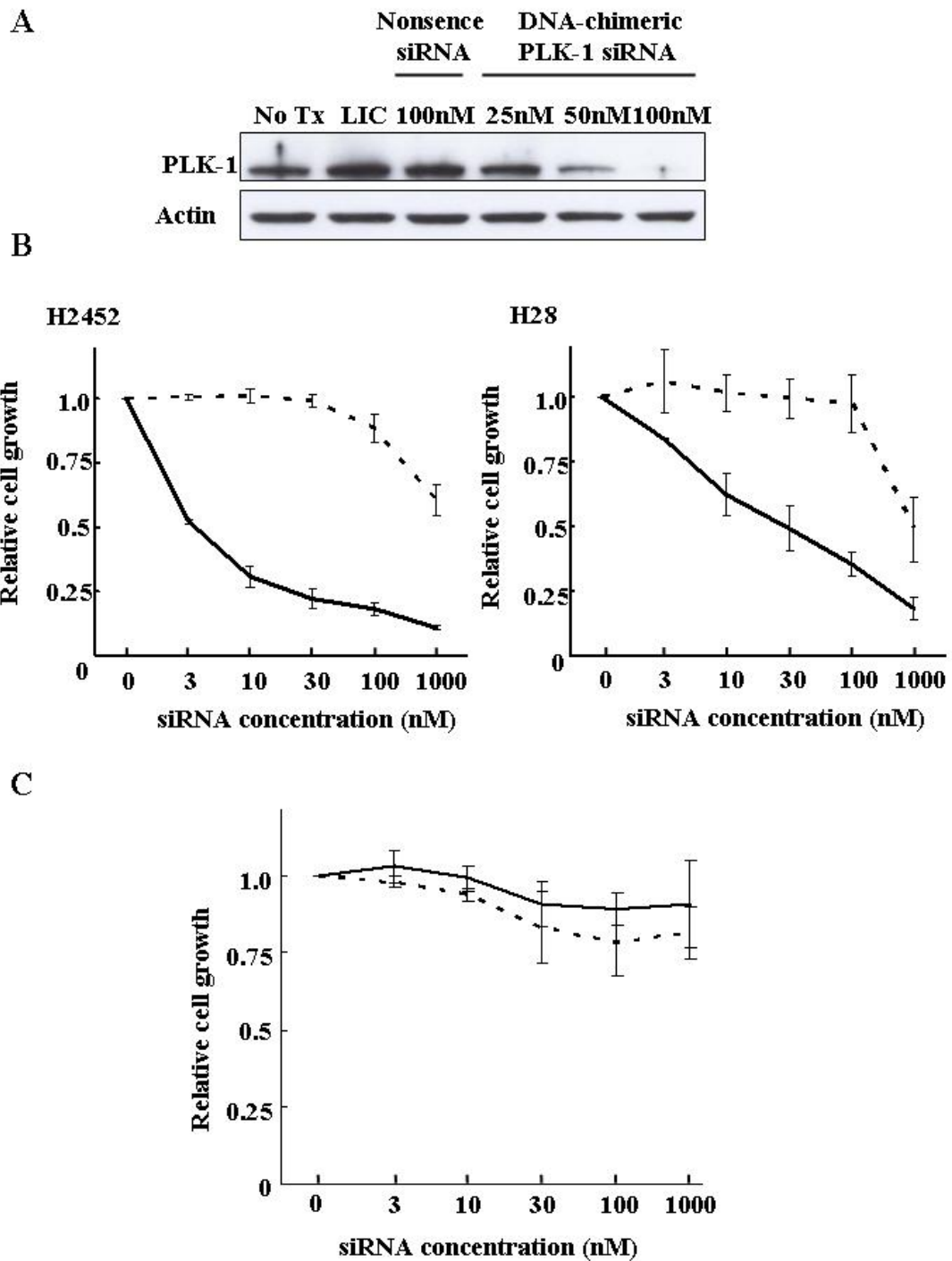
**Fig. 1.**



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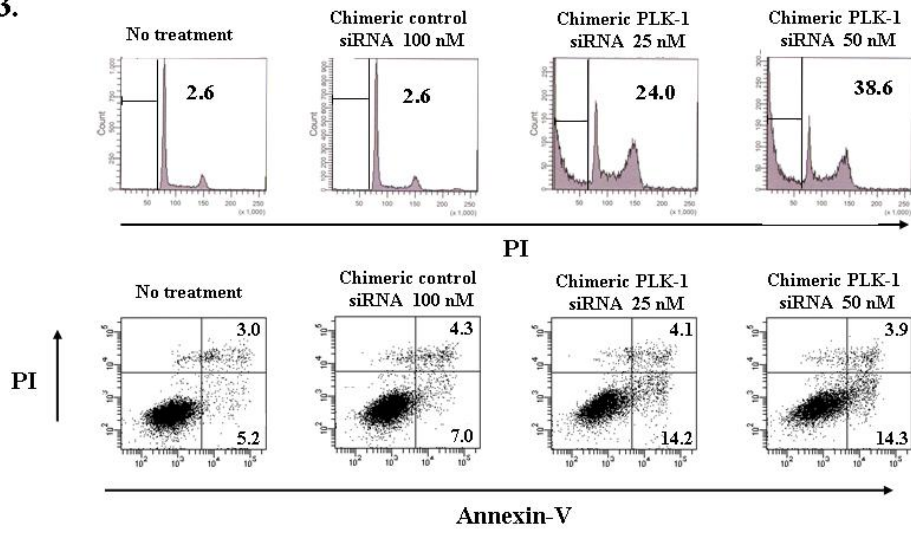
**Fig. 2.**



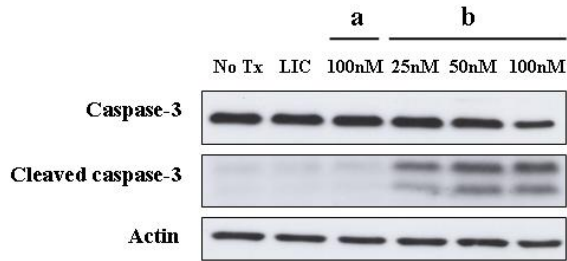
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**Fig. 3.**

**A**



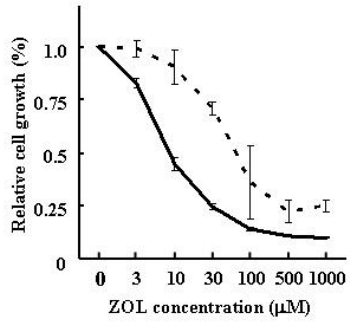
**B**



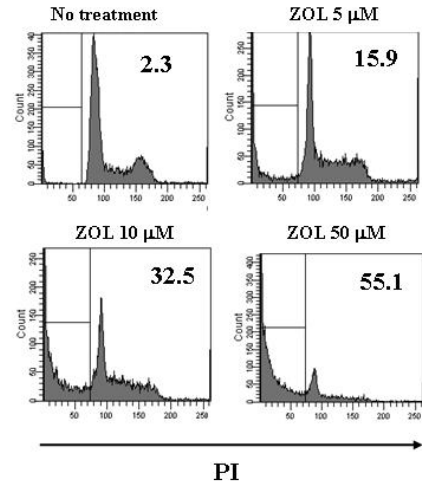
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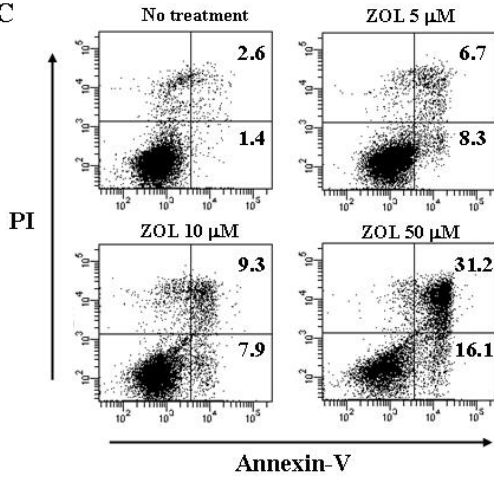
**Fig. 4.**  
**A**



**B**



**C**

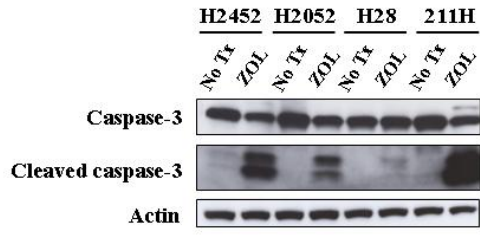


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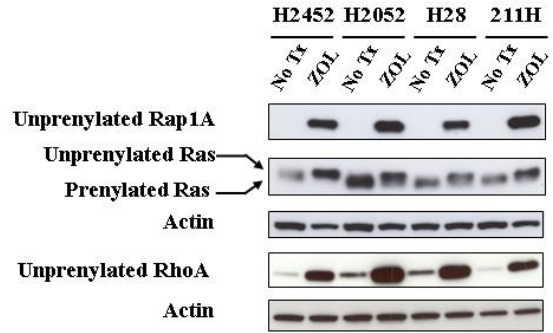
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Fig. 5.

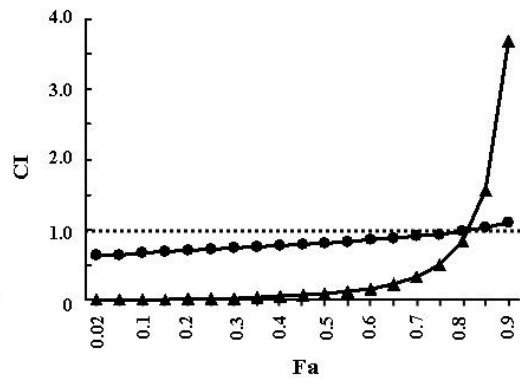
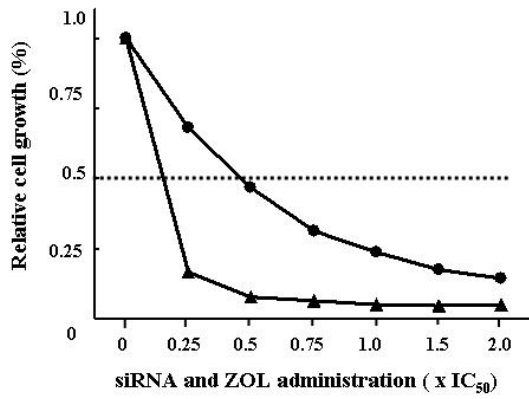
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C



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1 **Supplementary Figure legend**

2

3 **Supplementary Figure 1.** Evaluation of unprenylated RhoA expression in H2452 (left

4 panel) and H28 (right panel) mesothelioma cells by the treatment of PLK-1 siRNA

5 alone in combination with ZOL. Cells were incubated for 72 hours with PLK-1 siRNA

6 (50 nM) alone in combination with ZOL (50  $\mu$ M). Immunoblotting and

7 immunoprecipitation were performed as described in **Materials and Methods**.

8

**Supplementary Figure 1.**

