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Synergistic Antiproliferative Effects of Zoledronic Acid and Fluvastatin on Human Pancreatic Cancer Cell Lines: An in Vitro Study

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Bisphosphonates and statins are known to have antitumor activities against different types of cancer cell lines. In the present study, we investigated the antiproliferative effects of the combination of zoledronic acid (ZOL), a bisphosphonate, and fluvastatin (FLU), a statin, in vitro on two types of human pancreatic cancer cell lines, Mia PaCa-2 and Suit-2. The pancreatic cancer cell lines were treated with ZOL and FLU both individually and in combination to evaluate their antiproliferative effects using WST-8 cell proliferation assay. In this study, we demonstrated a potent synergistic antiproliferative effect of both drugs when used in combination in both cell lines. Moreover, we studied the molecular mechanism behind this synergistic effect, which was inhibited by the addition of the mevalonate pathway products, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Furthermore, we aimed to determine the effect of ZOL and FLU combination on RhoA and Ras guanosine 5′-triphosphate (GTP)-proteins. The combination induced a marked accumulation in RhoA and unprenylated Ras. GGPP and FPP reversed the increase in the amount of both proteins. These results indicated that the combination treatment impaired RhoA and Ras signaling pathway by the inhibition of geranylgeranylation and/or farnesylation. This study provides a potentially effective approach for the treatment of pancreatic cancer using a combination treatment of ZOL and FLU.

Key words  pancreatic cancer; zoledronic acid; fluvastatin; combination treatment; synergistic

Pancreatic cancer is known to be the fifth leading cause of cancer-related mortality in Japan and considered to be one of the most aggressive malignancies. Moreover, patients treated with surgical interventions usually develop tumor relapse and/or liver metastasis. Gene mutations, including K-ras, CDKN2A (p16), and TRP53, are often associated with pancreatic cancer. Previous studies have shown that blocking and/or depriving cells of K-ras may be a promising way to treat pancreatic cancer. Although gemcitabine has been considered the standard care treatment for pancreatic cancer, its clinical use remains limited. Therefore, the development of new therapeutic strategies is needed.

The third-generation nitrogen bisphosphonates (NBPs) are currently considered a key therapy for the treatment of osteoclast-mediated bone resorption, bone metastasis, and malignant skeletal-related diseases. NBPs have also shown direct antiproliferative and apoptotic effects on solid tumors by inhibiting the farnesyl pyrophosphate (FPP) synthase, a key regulatory enzyme in the mevalonate pathways. Impairing the prenylation of small guanosine 5′-triphosphate (GTP) proteins such as Ras, Rab, Rho, and Rac by FPP synthase inhibitors may lead to the loss of many cellular processes. Zoledronic acid is one of the most potent drugs owing to its direct and indirect antitumor effects, and it has been effectively used for the treatment of several cancer cell types such as breast and prostate cancers, acute promyelocytic leukemia, including pancreatic cancers. Moreover, ZOL was reported to have an augmenting effect when used with other antitumor agents, molecular target agents, and radiotherapy.

Statins have an inhibitory action on 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), a rate-limiting enzyme in the mevalonate pathway. Statins constitute a new anticancer therapeutic option because they exert cholesterol-independent effects. The antiproliferative effect of statins may act through inhibiting the synthesis of the mevalonate pathway byproducts, FPP and geranylgeranyl pyrophosphate (GGPP), which may lead to the impairment of small GTP proteins that play an essential role in carcinogenesis. Several studies have focused on the cytotoxic effects of fluvastatin (FLU), which inhibits cell proliferation, suppresses angiogenesis, and triggers apoptosis.

Although the antitumor effect of the combination of ZOL and FLU has been investigated in other types of cancer, it was not reported whether this combination treatment has a synergistic cytotoxic effect on pancreatic cancer cells or not. We evaluated the synergistic antiproliferative effect on two types of pancreatic cancer cell lines in vitro. In addition, we also sought to clarify the underlying molecular mechanism of the antiproliferative effect of the combination on the mevalonate-derived metabolites FPP and GGPP.

MATERIALS AND METHODS

Reagents  ZOL (Toronto Research Chemicals, Toronto, Canada) was dissolved in sterile distilled deionized water,
with a stock solution was prepared at 20 mM and stored at −20°C. FLU (LKT Laboratories, Inc., St. Paul, MN, U.S.A.) was prepared at 30 mM and diluted in dimethyl sulfoxide. Mevalonate isoprenoid metabolites, FPP (Sigma-Aldrich, Tokyo, Japan) and GGPP (Sigma-Aldrich) were used at a final concentration of 10 µM.

**Culture Media Conditions**  Human pancreatic cancer cell lines Mia PaCa-2 and Suit-2 were a kind gift from Dr. Soichi Takiguchi of the National Kyushu Cancer Center (Fukuoka, Japan). Cells were grown in RPMI-1640 media in 25 cm² tissue flasks supplemented with 10% fetal bovine serum (FBS), 1% glutamine, penicillin and streptomycin in a humidified atmosphere of 5% CO2 at a constant temperature of 37°C.

**Cell Viability Assay**  Cell suspensions were seeded into 96-well plates at the specified densities (3 × 10^3 cells/100 µL/well and 1 × 10^4 cells/100 µL/well for Mia PaCa-2 and Suit-2, respectively) and incubated at 37°C. Following a 24 h incubation period, cells were treated with different concentrations of ZOL and FLU. The treated cells were subsequently incubated for different time intervals of 24, 48, and 72 h. Cell viability was assayed using a Cell Counting Kit 8 (Wako Pure Chemical Industries, Ltd., Osaka, Japan). In brief, 20 µL tetrazolium solution (WST-8) was added to each well 1 h before the end of the experiment. The absorbance was recorded at 450 nm. Cell viability was determined from the absorbance of soluble formazan dye (orange color). The IC₅₀ was calculated using JMP software (version 11 pro; SAS Institute, Cary, CA, U.S.A.).

**Evaluation for Synergism or Antagonism**  To determine the degree of interaction between ZOL and FLU with Mia PaCa-2 and Suit-2 cells, the combination index (CI) was used. Based on the multiple drug effect equation of the Chou and Talalay method, the CI was calculated automatically using CompuSyn software (ComboSyn, Inc., Paramus, NJ, U.S.A.), where CI<1, CI=1, and CI>1 indicate synergism, additive, and antagonism, respectively.

**Western Blotting Analysis**  Cytoplasmic proteins in tumor cells (5 × 10⁵ cells/well) were extracted using CelLytic M Reagents (Sigma-Aldrich). The cells were homogenized in CelLytic 200 µL. The homogenate samples were centrifuged at 12000 rpm for 10 min. The supernatants were collected to new 600 µL tube. The supernatants were added 2× sample buffer and the mixtures were boiled at 99°C. The protein concentrations were determined using the BCA Protein Assay kit (Pierce Biotechnology, U.S.A.). Lysates (1 mg/mL) were separated using 10 or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were incubated with antibodies against Rhoa (67B9) (Cell Signaling Technology; #2117), Ras (Abcam; ab52939) and β-actin (Santa Cruz Biotechnology; SC-1616). The immunocomplexes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies anti rabbit immunoglobulin G (IgG)-HRP (Santa Cruz Biotechnology; SC-2030) and visualized using SuperSignal Chemiluminescent Substrate ImmunoStar® Basic (Wako). The membranes were photographed and the density of each band was analyzed using ImageQuant LAS-3000 (FUJIFILM, Tokyo, Japan) and ImageJ software.

**Statistical Analysis**  Each experiment was repeated at least three times, and all data are presented as the mean±standard deviation (S.D.). The statistical analysis was performed using JMP software. Statistical significance was evaluated using Dunnett’s test, which is used to compare the mean of each treatment group with that of the control. A pairwise comparison between groups was performed using the Tukey–Kramer (HSD) method. A statistically significant difference was expressed as p<0.05. Western blot analysis was performed using Tukey–Kramer (HSD) method.

**RESULTS**  Mia PaCa-2 cells were treated with ZOL (3–30 µM) and FLU (0.3–10 µM) and incubated for 24, 48, or 72 h to investigate the cytotoxic effect of each drug (Figs. 1A, B). ZOL and FLU, after 72 h incubation, inhibited the proliferation of Mia PaCa-2 cells in a dose-dependent manner, showing IC₅₀ values of 2.4 and 2.3 µM for ZOL and FLU, respectively.

Suit-2 cells were treated with ZOL (3–30 µM) and FLU (0.3–30 µM) and incubated for 24, 48, or 72 h. As shown in Figs. 1C and D, cell viability was significantly reduced in a dose-dependent manner (p<0.001), showing IC₅₀ values of 11 and 15 µM for ZOL and FLU, respectively.

We explored the effect of ZOL in combination with FLU on cell proliferation in Mia PaCa-2 cells. The concentration of ZOL remained fixed at 3 µM, while FLU was used at 0.3 and 1 µM for 24, 48, or 72 h (Fig. 2A). At 1 µM, FLU did not show a reduction of cell viability, and at 3 µM ZOL the cell viability was 51.2% in Mia PaCa-2 cells. However, a simultaneous treatment of 3 µM ZOL and 0.3 or 1 µM FLU for 72 h allowed cell proliferation by only 21.7±3.4, and 9.9±1.3%, respectively. At 72 h an IC₅₀ value of 0.2 µM was achieved when FLU was combined with ZOL, which is a much lower value compared to that of FLU (2.3 µM) when used individually.

When Suit-2 cells were treated with a combination of ZOL and FLU, an enhanced antiproliferative effect, compared with either single treatment, was observed (Fig. 2B). At 72 h incubation, simultaneous treatment with 3 µM ZOL and 3 or 10 µM FLU could reduce cell proliferation to 45.4±3.4 and 30.8±2.3%, respectively. An IC₅₀ value of 4.4 µM was achieved at 72 h when FLU was used in combination with ZOL, compared with that of 15 µM when FLU was used individually.

Moreover, the CI was calculated using CompuSyn software. Table 1 shows the CI values for each combination used in Mia PaCa-2 and Suit-2 cells, where all CI values were <1 (CI=0.60, 0.20, 0.31 in Mia PaCa-2 cells and CI=0.50, 0.35, 0.42 in Suit-2 cells) and consistent synergistic effects were obtained.

This experiment further elucidated the molecular mechanism by which ZOL and FLU induced their antitumor effects on pancreatic cancer cell lines. The inhibition of Mia PaCa-2 cell viability was restored from 12.5 to 83.7% with 10 µM ZOL and from 26.0 to 66.4% with 3 µM FLU (p<0.0001) after 72 h incubation with 10 µM GGPP (Fig. 3A). Similarly, a concomitant addition of FPP and GGPP restored the cell viability from 12.5 to 88.1% and from 26.0 to 88.3% (p<0.0001) for ZOL and FLU, respectively. On the other hand, FPP could only partially reverse the antiproliferative effect induced by FLU. Similar results were achieved with Suit-2 cells, where the addition of GGPP restored the cell viability from 46.3 to 85.1% and from 39.0 to 96.3% for ZOL and FLU, respectively (Fig. 3B).
Fig. 1. Antiproliferative Effect of ZOL and FLU on Pancreatic Cancer Cell Lines

The cytotoxic effect of a single treatment of (A) 3–30 µM ZOL, or (B) 0.3–10 µM FLU on Mia PaCa-2 cells, and (C) 3–30 µM ZOL or (D) 0.3–30 µM FLU on Suit-2 cells. Cell viability was calculated as a percentage of untreated cells (control). Data are expressed as the mean ± S.D. (n=3). Significant differences compared with the control are shown as **p<0.01, ***p<0.001 using Dunnett’s test.

Fig. 2. Synergistic Antiproliferative Effect of ZOL and FLU in Combination

(A) Mia PaCa-2 cells were treated with 3 µM ZOL combined with 0.3 or 1 µM FLU, and (B) Suit-2 cells were treated with 3 µM ZOL combined with 3 or 10 µM FLU for 24, 48, 72h. Significant differences between groups are shown as (**p<0.01 vs. control group, ##p<0.01, ###p<0.001 vs. ZOL using Tukey-Kramer’s HSD test. Data are expressed as the mean±S.D. of three triplicate experiments. IC_{50} value of FLU in combination with ZOL was calculated, where a single dose of ZOL was set as the control (100% cell viability).
To investigate the role of GGPP or FPP, cells were treated with a combination of ZOL and FLU and incubated with or without 10 \(\mu\)M FPP and/or 10 \(\mu\)M GGPP for 72 h. Figures 4A and B showed that GGPP almost completely rescued the inhibition of cell viability by the combination treatment; in Mia PaCa-2 the cell viability was restored from 12.4 to 85.1% and from 29.5 to 128.0% in Suit-2 cells. The addition of FPP could only achieve a partial reversal effect. On the other hand, the concomitant addition of FPP and GGPP restored the inhibition of cell viability to the control level (95.4 and 98.9% in Mia PaCa-2 and Suit-2 cells, respectively). However, the addition of FPP or GGPP alone showed no significant difference compared with the control.

To further investigate whether the synergistic antiproliferative effect of ZOL and FLU combination treatment is due to the inhibition of RhoA and/or Ras proteins isoprenylation upon the decrease of FPP and/or GGPP synthesis. Suit-2 cells were incubated with 3 \(\mu\)M ZOL and/or 10 \(\mu\)M FLU for 24 h and the amount of RhoA protein (Fig. 5A) and the ratio of unprenylated Ras protein to prenylated one (Fig. 5B) were examined using Western blot analysis. ZOL-treated cells showed no effect. FLU treated cells resulted in an increase in the amount of RhoA protein and the ratio of unprenylated Ras protein.

<table>
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<tr>
<th>Cell line</th>
<th>Drug combination</th>
<th>CI</th>
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<tr>
<td>Mia PaCa-2 cells</td>
<td>i. ZOL (1 (\mu)M)+FLU (1 (\mu)M)</td>
<td>0.60</td>
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<td>ii. ZOL (3 (\mu)M)+FLU (0.3 (\mu)M)</td>
<td>0.31</td>
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<td>iii. ZOL (3 (\mu)M)+FLU (1 (\mu)M)</td>
<td>0.20</td>
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<tr>
<td>Suit-2 cells</td>
<td>i. ZOL (1 (\mu)M)+FLU (3 (\mu)M)</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>ii. ZOL (3 (\mu)M)+FLU (3 (\mu)M)</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>iii. ZOL (3 (\mu)M)+FLU (10 (\mu)M)</td>
<td>0.42</td>
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The CI was calculated using CompuSyn software, where a CI value <1 indicates synergy, CI=1 additive and CI>1 indicates antagonism.

When cells were treated with a combination of ZOL and FLU, marked increases in the RhoA protein and unprenylated Ras protein was observed. To determine which mevalonate isoprenoid metabolite acts as a key regulatory for RhoA protein and unprenylated Ras protein, Suit-2 cells were incubated with a combination of ZOL and FLU in presence of 10 \(\mu\)M FPP or 10 \(\mu\)M GGPP. FPP could partially reverse the effect of the combination treatment on the amount of RhoA, and completely decrease the ratio of unprenylated Ras. GGPP significantly restored the both proteins to their basal levels.

**DISCUSSION**

This study highlighted the synergistic antiproliferative effect resulting from the combination of ZOL with FLU in two pancreatic cancer cell lines. A previous study had showed the efficacy of ZOL in pancreatic cancer cell lines. Our present study showed that ZOL significantly induced antiproliferative effects in Mia PaCa-2 and Suit-2 pancreatic cancer cell lines in a dose- and time-dependent manner. We also showed that FLU can induce a significant antiproliferative effect in pancreatic cancer cell lines, which is in good accordance with previous studies, and confirmed them as highly effective and reliable chemotherapeutic agents.

Many studies have explored the effect of statins and/or bisphosphonates in combination with other drugs on different types of cancer cell lines. Synergistic cytotoxic effects were reported between FLU and gemcitabine against pancreatic cancer cells, and FLU and trastuzumab against breast cancer, and FLU and cisplatin against ovarian cancer cells. In addition, a combination of ZOL and gemcitabine was shown to have a potentiated anticancer effect and it was reported to inhibit liver metastasis in pancreatic cancer. Other studies have also shown that panobinostat, a histone deacetylase inhibitor, induced a synergistic cytotoxic effect when combined with ZOL against prostate cancer and multiple myeloma.
Fig. 4. The Effect of Mevalonate-Derived Products on ZOL and FLU Combination-Induced Antiproliferative Activity in Pancreatic Cancer Cell Lines

(A) Mia PaCa-2 cells were incubated with a combination of 3 µM ZOL and 1 µM FLU with or without 10 µM FPP and/or 10 µM GGPP for 72 h. (B) Suit-2 cells were incubated with a combination of 3 µM ZOL and 10 µM FLU with or without FPP and/or GGPP for 72 h. Data are expressed as the mean ± S.D. (n = 3) and significant differences are shown as ***p < 0.001 vs. control, **p < 0.01, ###p < 0.001 vs. ZOL+FLU using Tukey-Kramer’s HSD test.

Fig. 5. The Effect of ZOL and FLU Combination on the Amount of RhoA and the Ratio of Unprenlated Ras Proteins to Prenylated One in Suit-2 Cells

Suit-2 cells were incubated with 3 µM ZOL and/or 10 µM FLU with or without 10 µM FPP and 10 µM GGPP for 24 h. Cell lysates were extracted and incubated with antibodies against RhoA (A) and Ras (B) and analyzed by Western blot. Quantitative densitometric comparison of the results shown in a expressed as the mean ± S.D. Results normalized to β-actin and expressed as the mean ± S.D. (n = 3) (A). *p < 0.05, as compared to controls and each single treatment Tukey-Kramer’s HSD test.
Our study is the first analysis of combination treatment with ZOL and FLU using Mia PaCa-2 and Suit-2 pancreatic cancer cells. The results showed a clear synergistic antiproliferative effects with CI values of <1. In addition, the combination treatment could reduce the FLU IC\textsubscript{50} values from 2.3 to 0.2 µM in Mia PaCa-2 cells and from 15 to 4.4 µM in Suit-2 cells compared with FLU used individually. This synergistic antiproliferative effect of ZOL and FLU also agreed with a previous study that showed a potentiated cytotoxic effect of this combination in T24 bladder cancer cells.\textsuperscript{40) Moreover, it was reported that a combination of ZOL and FLU had a high safety margin and possessed no potential for drug interactions when used with other cancer cell lines.\textsuperscript{30,41,42)\n
There are controversies over the inhibition mechanism of the mevalonate pathway by NBPs and statins. The molecular mechanism of NBPs is usually due to the inhibition of FPP synthase and loss of signaling in the downstream pathway of small GTPases, such as Ras proteins.\textsuperscript{16,43) Previous studies showed that a consequence of FPP synthase inhibition by ZOL is the accumulation of intracellular isopentenyl pyrophosphate (IPP), which leads to the formation of pro-apoptotic ATP analogue APPPI (triphosphoric acid 1-adenosin-5’-yl ester 3-(3-methylbut-3-enyl)ester) inducing apoptosis\textsuperscript{44,45) (Fig. 5). Moreover, other studies documented that the antitumor effect of ZOL on prostate cancer cells is due to blockage of the geranylgeranylation of Rho proteins rather than farnesylation.\textsuperscript{46) On the other hand, the cytotoxic and antiproliferative effects of statins have been attributed to the impairment of geranylation suggesting that GGPP, not FPP, is the main target for their anticancer effect.\textsuperscript{47) However, other studies reported that it is more plausible that inhibition of FPP synthase will subsequently prevent farnesylation and geranylation of GTP proteins.\textsuperscript{48,49) These contradictory data reflect that the actual molecular mechanisms are still not fully understood.

We also sought to investigate the molecular mechanism by which ZOL and FLU induce their antiproliferative effect in pancreatic cancer cells. We showed that the co-treatment with GGPP completely reversed the inhibition of ZOL on pancreatic cancer cell lines (Figs. 3A, B). On the other hand, the addition of FPP could not inhibit the antiproliferative action of ZOL. This mechanism mainly may depend on the IPP/APPPI accumulation. The addition of GGPP but not FPP into ZOL-treated cells resulted in an almost complete inhibition of IPP accumulation through the down regulation of HMG-CoA reductase in addition to rescuing small GTPases prenylation.\textsuperscript{37,50) However, the difference in the ability of FPP to restore the cell viability in case of the combination treatment (3 µM ZOL+1 µM FLU) and the single treatment of 3 µM FLU could be due to the different FLU concentration used in each case (Figs. 3A, 4A). We assumed that 3 µM FLU inhibited the synthesis of IPP more strongly than 1 µM FLU leading to a lower level of GGPP that was converted from the added FPP. In Suit-2 cells, FPP could only partially rescue their antiproliferative effect of 10 µM FLU (Fig. 3B) and the combination of 10 µM FLU and 3 µM ZOL (Fig. 4B). It is expected that the concentration of FLU inhibited the synthesis of IPP so only a small amount of IPP was available to convert FPP into GGPP. Therefore, we can hypothesize that FLU concentration is an important factor in the blockage of the FPP conversion into GGPP. However, we did not determine the level of IPP/APPPI or FPP/GGPP in this study, and further investigations will be required in the future.

Since prenylation of small GTP-proteins of Rho and Ras plays an important role in the development and progression of pancreatic cancer,\textsuperscript{51) we attempted to evaluate whether the synergistic antiproliferative effect of ZOL and FLU combina-

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**Fig. 6. A Modified Schematic Summary of the Mevalonate Pathway Involved in the Synergistic Antiproliferative Effect of ZOL and FLU in Combination in Pancreatic Cancer Cells**\textsuperscript{40)
tion is responsible for the inhibition of RhoA and/or Ras prenylation. First, we showed that the combination increased the amount of RhoA protein compared to the control (Fig. 5A). However, we did not separate the prenylated and unprenylated RhoA protein. It was reported that lovastatin and ZOL induced an accumulation of RhoA protein and functionally active RhoA was significantly reduced in Human Trabecular Meshwork Cells and in Saos-2 osteoblast-like cells, respectively. We speculate that ZOL and FLU combination induced the accumulation of RhoA protein and impaired RhoA membrane localization inhibiting its prenylation and activation. In addition, the combination treatment resulted in a slower migration band and accumulation of the unprenylated Ras protein (inactive form) in Western blotting analysis. Furthermore, FPP and GGPP could repress the increase in RhoA and unprenylated Ras protein. Our present study showed that the synergistic antiproliferative effect of ZOL and FLU combination treatment is mediated through the inhibition of RhoA and/or Ras protein prenylation, which is due to the inhibition of geranylgeranylation and/or farnesylation (Fig. 6). However, this mechanism of action is still unclear and need further investigations.

Therefore, we can assume that GGPP is an important target for the synergistic antiproliferative effect, which agrees with a previous study that showed similar results when the same drugs were used against bladder cancer cell lines. However, we cannot ignore the role of FPP as an essential metabolite that was also required for the partial rescue of pancreatic cancer cell growth. Furthermore, due to the similar results obtained in both Mia PaCa-2 and Suit-2 cell lines, we can infer that the mechanism of action of ZOL and FLU was not cell-type specific.

It should also be taken into consideration that synergistic cytotoxic effect of pamidronic acid (PAM), a bisphosphonate, and lovastatin (LOV), a statin, have been investigated before against pancreatic cancer cell lines. They evaluated the mechanism responsible for the anti-tumor effect induced by this combination mainly on Panc-2, murine pancreatic carcinoma cell lines. Panc-2 cell line lacks clinical significance for pancreatic cancer due to absence of mutational spectrum when compared to human diseases, which limited its clinical application. In our study, we investigated the mechanism responsible for the synergistic antiproliferative effect of ZOL and FLU combination against human pancreatic cancer cell lines including metastatic one (Suit-2) providing a more realistic data for clinical applications. Moreover, in our study, the drug concentrations used against Mia PaCa-2 cells were much lower than those applied in the previous study. After 48h incubation, the combination 3 µM ZOL with 0.3 µM FLU in our study and that of 20 µM PAM with 2 µM LOV reduced the cell viability by about 50%. In addition, FLU has an excellent bioavailability, lower potential for drug–drug interactions, fewer side-effects and a better safety profile compared with LOV. ZOL was shown to have a shorter infusion time, better efficacy and safety profiles compared to PAM. Therefore, comparing our combination treatment to that of PAM and LOV, we expect that the combination treatment of ZOL and FLU, if explored clinically, might offer a more effective strategy in the treatment of pancreatic cancer cases.

In conclusion, this is the first in vitro study that demonstrates the synergistic antiproliferative effect of ZOL and FLU in combination treatment in pancreatic cancer cell lines. We suggested that the molecular mechanism for this synergistic effect acts through the impairment of prenylation of RhoA and/or Ras proteins. In addition, ZOL and FLU have been used clinically and are known to have few side-effects that are well tolerated. Taking the present data into account, we suggest that the combination of ZOL and FLU may represent a promising treatment for pancreatic cancer in the future. Hereafter, an in vivo study is needed to better understand the antiproliferative effect of this combination treatment.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES


13. Van Beck E, Pieterman E, Cohen L, Løwik C, Papappoulos S. Farnesyl pyrophosphate synthase is the molecular target of nitrogen-


42) Dunford JE, Thompson K, Coxon FP, Luckman SP, Hahn FM, Poulter CD, Ebetino FH, Rogers MJ. Structure–activity relationships for


