Referee 1

General

In the present study, authors investigated the effects of YM529 in preventing the growth of non-small cell lung cancer (NSCLC) cells in vitro. In vitro, YM529 dose-dependently inhibited the growth of different bladder cancer cell lines possibly through the inhibition of phospho-ERK1/2. In addition, YM529 showed direct anti-tumor effect on NSCLC. The authors conclude that YM529 may be a potent anti-cancer agent for NSCLC. I have several comments.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached).

Authors show the growth inhibitory effect of YM529 against NSCLC cells. However, many previous reports have already demonstrated similar results to this manuscript using various types of cell lines. There seems to be little additional and important information.

Response: The main purpose of this study is to examine the effect of third generation BP (YM529) on non-small cell lung cancer (NSCLC). As the reviewer pointed out, the effect of third generation BP like YM529 and ZOL has been studied in various cancer cell types but the effect is different according to cancer type. Drugs are not effective for some kinds of cancer cell lines including myeloma and leukemia. Thus we do not think that our study that demonstrate the efficacy of YM529 on 8 NSCLC cell lines is little additional and important information, but believe that our study is a pivotal finding for further study such as in vitro and combination studies with other cytotoxic drugs that were shown in SCLC.

Moreover, these experiments are immature. Although authors did not demonstrate any Ras-contribution, authors concluded this growth inhibitory effect is due to the reduction of Ras-signal.

Response to this comment and related Minor Essential Revisions 6 comment:

It is well known that the property of BP including YM529 is to inhibit Ras in osteoclast. We presented the western blotting for pan-Ras protein to show that the Ras was expressed in same level in treated cell lines. Our focus is whether downstream molecule p-ERK1/2 is actually inhibited by YM529 or not, which may be
more important than Ras issue for this paper not only because as above reason but p-ERK1/2 is more directly related to apoptosis. Regarding unprenylated and prenylated forms of Ras in western blotting, as the reviewers comment, two bands should be separated. Indeed, this finding was shown in several papers from the reviewer and his colleagues (Kuroda et al. Blood 102, 2229-35, etc). The reason why our western blotting seems to be one band may be that we used the 10% SDS-PAGE of which polyacrylamid % might be too low for its molecular weight (21 kDa) to have separate bands. Tassone et al. showed that western blotting for Ras with single band (there was no information for concentration of gel) (Tassone et al, British J Cancer 88,1971-1978). However, we will retry western blotting with higher concentration gel if reviewer still think that the Ras western blotting with unprenylated and prenylated forms is "essential" for this paper.

For this revise, to prevent confusing Ras issue, we delete Ras western blotting figure and make some corrections as described below.

P10 line 16,
We change "Western blotting analysis revealed no remarkable changes in the expression of Ras or..." to "Western blotting analysis revealed no remarkable changes in the expression of pan-Ras (data not shown) or..."

P12 line 13.
A sentence “This mechanism was demonstrated by analysis using NCI-H1819 cell lines.” is deleted.

P13 line 10.
We change "Because YM529 inhibits farnesylation..." to "Because YM529 is assumed to inhibit farnesylation...".

Moreover, it is critical to know whether the levels that were achieved in these NSCLC cells in vitro are ever likely to be achievable in humans. In order to let the readers to better understand their speculation that YM529 is useful for the patients with NSCLC, authors should show the growth inhibitory effect against the tumor bearing mice in vivo. I think this is the initial step for the translational research.

As reviewer's comment, pharmacokinetics of YM529 in human is very important for clinical application. We are planning to examine in vitro model based on our findings shown in this study.

----------------------------------------------------------------------------------------------------------------------

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

1 As opposed to what is claimed in the introduction section (page 5), a previous study has demonstrated that ZOL reduced tumor growth of human squamous carcinoma in the tibiae of treated mice (Tannehill-Gregg SH et al., 2006). The authors should discuss this previous study along with their own data.

We appreciate this comment: We change our description: "However, study that evaluated the effect of BPs including YM529 on NSCLC has been limited and the effect of zoledronic acid on only one cell lines was examined." and we cite this reference.
2 page 4 and page 11: what is the R2? Although it might be a common word among the bisphosphonate investigators, I think it should be the alternative common name or needs elucidation.

We delete R2 and added following description. "BPs have a common basic structure and different substituents at the one of two covalentlybounded side-chain (R2) attached to the germinal carbon, which strongly influence their pharmacologic properties."

3 In the abstract, page 2, (in vitro or/and in vitro) should be (in vitro or/and in vivo)?

It is our mistake and we correct this part.

4 page 4, (classified) should be classified.

We correct this part.

5 The elucidation, YM529 (1-Hydroxy-2-imidazo....) should not be duplicated (page 4, 5) Dot (.) is not necessary before Equal (page 8).

We deleted duplicated part for YM529 in page8 (Material and Methods).

6 In the Western blotting analysis using Ras antibody, there should be two bands which indicate unprenylated and prenylated forms of Ras. The prenylated and unprenylated forms of Ras move differently in the gel electrophoresis. Moreover, the activated and prenylated forms of Ras should be in the membranous fractions, whereas inactivated and unprenylated forms of Ras should be in the cytoplasmic fraction. I think authors can show the growth inhibition of NSCLC cells by YM529 would be due to Ras dependent pathway or not. I could understand author speculated cellular apoptosis was induced thorough the reduction of the phosphorylation of the ERK1/2 because phosphoERK1/2 was reduced after YM529 treatment. However, authors did not show that YM529 inhibited the prenylation of Ras. I think the authors should investigate whether the alteration of ERK1/2 signal is Ras-dependent or Ras-independent pathway. Separation of membrane and cytoplasmic fractions is one way to show it.

Response was described above. We appreciate this suggestion and kind understanding what we intend to show in this study.

Thank you so much for review work.

Referee 2

General

R Koshimune et al present an interesting report on the anti-tumor activity of novel bisphosphonate (YM529) on non-small cell lung cancer cell lines. The authors results provide evidence that the compound indeed merits further development as anticancer agent. The experimental work is well designed and results are clear.
Response: We really appreciate this comment and high evaluation for our work.

---------------------------------------------------------------

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached).

---------------------------------------------------------------

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

pag 2, line 6-7 from top: in vitro and in vitro ?

We correct this part.

Thank you so much for review work.