Author's response to reviews

Title: Antitumor activities of ATP-competitive inhibitors of mTOR in colon cancer cells

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Author's response to reviews: see over
Dear Editor,

Please find enclosed a revised version of our manuscript entitled “Anti-tumor activities of ATP-competitive inhibitors of mTOR in colon cancer cells”. We are grateful to both reviewers for their very clear comments. We were pleased to see their positive comments and we have carefully followed their recommendations to improve the quality of our manuscript.

Reviewer 1:

Major Compulsory Revisions:

1) “It's not clear why the three colon cancer cell lines (LS174T, DLD-1 and SW480) are particularly chosen in this study. A large panel of colon cancer cell lines is needed to confirm the sensitivity in response to the treatment of PP242 and rapamycin. In addition, do PP242 and NVP-BEZ235 also effectively inhibit DLD-1 xenograft tumor growth as they did in LS174T and SW480 (Fig. 3)?”

We initially wanted to test the efficacy of mTOR inhibitors on colon cancer cells harboring distinct mutations of PI3KCA. Most of the time mutations arise at the helical domain (exon 9) or the kinase domain (exon 20) of the catalytic subunit of PI3K. LS174T cells have a PI3KCA mutation on exon 20 and DLD-1 on exon 9. In contrast, SW480 cells have no PI3KCA mutation. Our results suggest that the in vitro antitumor efficacy of ATP-competitive inhibitors of mTOR do not depend on the PI3KCA status of colon cancer cells. This explanation was added in the results section of the manuscript and the following paragraph was also added in the discussion.

“We initially hypothesized that ATP-competitive inhibitors of mTOR would produce anticancer activity only in cells harboring PI3KCA mutations. To support this hypothesis it was previously reported that NVP-BEZ235 was effective in PI3K but not in KRAS mutated breast cancer cells and similar findings were reported in a murine model of lung cancer {Brachmann, 2009 #61} {Engelman, 2008 #62}. However, we observed here that ATP-competitive inhibitors of mTOR...
exhibited anticancer effects on both \(PI3KCA\) mutated as well as on \(PI3KCA\) wild type colon cancer cells. Consistent with our findings, NVP-BEZ235 is effective in a mouse model of sporadic \(PI3KCA\) wild type CRC suggesting that the antitumor activity of ATP-competitive inhibitors of mTOR is not restricted to \(PI3KCA\) mutated colon cancer cells (Roper, 2011 #54).”

As suggested by the reviewer we tested a larger panel of colon cancer cells. In addition to LS174T, DLD-1 and SW480 we also tested the effects of mTOR inhibitors on Caco-2 (\(PI3KCA\) wild type), SW620 (\(PI3KCA\) wild type), HT29 (\(PI3KCA\) wild type) and HCT-116 (\(PI3KCA\) mutated) colon cancer cells. We found that PP242 and NVP-BEZ235 constantly reduced colon cancer cell growth. Rapamycin diminished growth of HT29 and HCT-116 cells but had no effect on Caco2 and SW620 cells. These results appear in a new Additional File 1.

We did not test the efficacy of ATP-competitive inhibitors of mTOR on DLD-1 cells as we were limited by the number of animals that we were allowed to use for this study. We chose LS174T and SW480 cells to compare the efficacy of mTOR inhibitors on \(PI3KCA\) mutated versus \(PI3KCA\) wild type xenografts.

2) **PP242** can effectively inhibit both mTORC1 and mTORC2 at 100nM as evidenced by the dephosphorylation of S6 and AKT, respectively (Fig. 1), but why did authors use 10 \(\mu\)M of PP242 and 1 \(\mu\)M of NVP-BEZ235 in the cell proliferation and survival studies (page 5, line 4 from bottom)? Using 10 \(\mu\)M of PP242 may cause off targets such as to PI3K, PKC and others (PLoS Biology, 2009, 7(2):e38).

We agree and repeated all the experiments with the lowest concentrations that effectively inhibited mTORC1 or mTORC2 as observed by Western Blot (10 nM of rapamycin, 100 nM of NVP-BEZ235 and 100 nM of PP242). We made similar observations than with higher concentrations though the effects were less pronounced. These new results are presented in Figure 2 and 4.

3) A recently published paper (PLoS One, 2011, 6(9):e25132) showed that NVP-BEZ235 (500 nM) inhibits cell proliferation but has no effect on apoptosis as determined by cleaved PARP in DLD-1 and SW480 cells, whereas authors showed in the Fig.2 that NVP-BEZ235 (1 \(\mu\)M) induced apoptosis in the same two cell lines as assessed by a cell death detection ELISA. Is the difference because of off target and/or using different methods for the detection? It’s better to combine other methodologies such as FACS analysis to confirm this difference.

Using lower concentrations of NVP-BEZ235 and PP242, we still found that both compounds induce colon cancer cell apoptosis by using the cell death detection ELISA. This suggests that mTOR inhibition is responsible for this effect rather than an “off target” phenomenon. As proposed by the reviewer we analyzed the percentages of sub-G1 population in colon cancer cells following treatments by flow cytometry and obtained similar results as with the ELISA. These results are presented in a new additional file 2. We think therefore that the difference obtained with previous findings (PLoS One, 2011) relies on the different methods used to detect apoptosis. The flow cytometry analysis shows that, although the difference is statistically significant, the pro-apoptotic effects of PP242 and NVP-BEZ235 are modest. We therefore think that this difference might be overlooked by Western blot. In fact, we also did not detect cleaved caspase-3 by Western blot in our conditions.

4) In Fig. 4, what is the mechanism underlying the synergistical antitumor activity by combination of the mTOR kinase inhibitors (PP242 or NVP-BEZ235) and the MEK inhibitor (UO126) regardless of
whether mTOR inhibitors increase pMAPK? Can UO126 effectively inhibit pMAPK in vivo? Does the combination of both drugs inhibit cell proliferation and induce apoptosis in vivo as evidenced, for instance, by Ki-67, cleaved PARP or TUNEL?

As proposed by the reviewer we investigated the mechanisms responsible for the synergistical antitumor activity of combined mTOR and MEK inhibitions. By Western Blot analysis, we found that U0126 effectively inhibits pMAPK in vivo. In addition we also observed that combined inhibitions in contrast to single therapies also induce the expression of cleaved caspase-3. Finally, using Ki-67 stainings, we also observed that the anti-proliferative activities of combined therapies are bigger than either treatment alone. Therefore this suggests that the mechanism underlying the synergistical antitumor activity by combining mTOR and MEK inhibitors involved both induction of apoptosis and stronger anti-proliferative effects. These results are now presented in a new figure 5.

Minor essentials Revisions:

1) The references are needed for the preparation of PP242, NVP-BEZ235, rapamycin and UO126 in in vivo study (page 5, line 10 from bottom).

As also required by reviewer 2, we added the details for the preparation of PP242, NVP-BEZ235, rapamycin and U0126 in the material and methods section

2) In Fig. 1, one of cell lines is indicated as SW620, which should be corrected to SW480.

   We changed SW620 to SW480

Reviewer 2:

Major Compulsory Revisions:

1) In Fig. 1, the effects of rapamycin, PP242 and NVP-BEZ235 on p-Akt and p-S6 were compared at same concentrations (0, 10, 100, and 1000 nM). At 100 nM, all three compounds were able to completely inhibit mTORC1 (p-S6), and only rapamycin activated p-Akt in LS174T, SW480 and DLD-1 cells. However, in Fig. 2, different concentrations of rapamycin (100 nM), NVP-BEZ235 (1µM), and PP242 (10 µM) were used to compare their effects on cell growth, BrdU labeling and DNA fragmentation. One would wonder whether the “superior” anticancer effects of PP242 and NVPBEZ235 are due to the higher concentrations used. It is good to compare their effects at the same concentration range as in Fig.1, given that the anticancer effect is attributed to inhibition of mTOR. This is applicable to Fig.4 as well.

   As mentioned previously, we totally agree and repeated the experiments with the lowest concentrations that effectively inhibited mTORC1 or mTORC2 as observed by Western Blot (10 nM of rapamycin, 100 nM of NVP-BEZ235 and 100 nM of PP242). We made similar observations than with higher concentrations though the effects were less pronounced. These new results are presented in Figure 2 and 4.

2) In Fig.3, the dose (1.5 mg/kg/day, i.p.) of rapamycin for in vivo studies was much lower than that (5-20 mg/kg/day, i.p.) in other reports (e.g. Clin Cancer Res. 2001, 7:1758-64; Mol Cancer Ther. 2009,8:2255-65). Therefore, it is hard to say that PP242 and NVP-BEZ235 are really superior to
rapamycin in inhibition of the xenografts growth in nude mice. To make the story convincing, it is necessary to use a commonly accepted dose (at least 5 mg/kg/day rapamycin, CCI-779 or RAD001, i.p.) for the in vivo studies.

We totally agree that the dose of rapamycin that we used was lower than in other reports. However as we previously published that rapamycin at 1.5mg/kg/day inhibited mTORC1 in xenografts (Abdelnour-Bechtold et al, Anticancer Res 2010; Benoit M et al J Surg Res 2011), we had to use this concentration in the animal protocol used for this study. In addition, we also found here that 1.5 mg/kg/day blocked mTORC1 activity as observed by Western blot (Figure 3). This suggests that at least at concentrations that inhibit mTORC1 activity, ATP-competitive inhibitors of mTOR are superior to rapamycin. Similar to our findings Cho et al reported that in renal cell carcinoma the anticancer efficacy of NVP-BEZ235 was superior to rapamycin when used at 3.5 mg/kg/day. (Cho et al Clin Cancer Res 2010, 16(14):3628-38).

We however agree that further studies are needed to compare ATP-competitive inhibitors of mTOR and higher doses of rapamycin before drawing any conclusion. We have therefore dampened our conclusions and added the following chapter in the discussion:

“The anticancer efficacy of NVP-BEZ235 and PP242 was both in vitro and in vivo superior to rapamycin. It is however worth noting that despite blocking mTORC1 activity in vivo, the doses of rapamycin that we used (1.5 mg/kg/day) were lower than those reported by other groups (5 mg/kg/day and 20 mg/kg/day) [Dudkin, 2001 #63] [Ekshyyan, 2009 #64]. Therefore a comparison between ATP-competitive inhibitors of mTOR and higher concentrations of rapamycin is needed to conclude that ATP-competitive inhibitors of mTOR are more efficient than rapamycin. Nevertheless, similar to what we found, it was reported in renal cell carcinoma, that the anticancer efficacy of NVP-BEZ235 was superior to rapamycin used at 3.5 mg/kg/day [Cho, 2010 #47].”

Minor essentials Revisions:

1) All abbreviations should be defined at the first appearance.

We have added all abbreviations at the first appearance.

2) It is not clear how rapamycin, PP242 and NVP-BEZ235 were prepared for in vitro and in vivo studies. This should be described.

We have added it in the material and methods section.

3) In Fig.4B and C, the concentration of U0126 should be given.

We added the concentration of U0126 which was 10 µM in the legend of figure 4.

4) Please correct “UO126” to “U0126”

U0126 was corrected to U0126.

5) Please check typos, e.g. “UO126 (40 lmmol/kg/d, i.p.)” (Page 5).

We have checked the typos throughout the text.
Editorial requirements:

Competing interests and authors’ contribution have been added. The ethical committee for animal ethics approval is the cantonal veterinary office of Canton Vaud and has been added in the material and methods section.

We hope that you will find our revised manuscript satisfactory for publication and are looking forward to hearing from you.

Sincerely,

Olivier Dormond MD. PhD.