Author's response to reviews

Title: The Rho-kinase inhibitor HA-1077 suppresses proliferation/migration and induces apoptosis of urothelial cancer cells

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Author's response to reviews: see over
On February 16, 2014

Dear Dafne Solera

Executive Editor, BMC Cancer

Enclosed please find a revised version of our manuscript (Manuscript # 6805191881001469) entitled "The Rho-kinase inhibitor HA-1077 suppresses proliferation/migration and induces apoptosis of urothelial cancer cells".

We appreciate the thoughtful constructive suggestions of the reviewers.

We have responded to each point on accompanying page.

We have performed additional statistical analysis, according to the comment of three reviewers. The biggest change in this revised version is that we examined the difference in the percentage of proliferated, apoptotic or migrated cells at each HA-1077 concentration was compared between cultures with HA-1077 alone and cultures with HA-1077 plus LPA and GGOH. Similarly, in this revised study, we also examined the change of Rho activity and expression of ROCK-I and ROCK-II between cultures with HA-1077 alone and cultures with HA-1077 plus LPA and GGOH. Therefore, our conclusions in this revised manuscript that HA-1077 prevents the proliferation and migration of bladder cancer cells and also induces apoptosis by inhibiting ROCK may be more accurate than our conclusions in the previous version.

We also reorganized the manuscript by having the paper looked at by native English speaker.

We changed the title like below.

"The Rho-kinase inhibitor HA-1077 suppresses proliferation/migration and induces apoptosis of urothelial cancer cells".

We added one author, Hiromichi Shirataki, and deleted previous first author, Nobutaka Furuya, and Hideyuki Abe was new first author in this revised version.

We are willing to pay for the printing in our article.

The comments offered by three reviewers have been helpful in formulating what we believe is a stronger paper. We appreciate these thoughtful comments, and hope that our responses and in particular our revisions have allowed this paper to achieve a priority sufficient for publication in BMC Cancer.

Sincerely yours,

Takao Kamai, M.D., PhD.

Reply to reviewer 1: Dr Bharat Joshi

Major compulsory revisions:

The answer to 1

We appreciate the thoughtful constructive suggestions of the reviewer.

We understand the necessary of investigation of in vivo animal model to explore the roles of HA-1077 in cancer treatment, however, at present, we can not afford instrument
and device to run experiment using *in vivo* animal model. Thus, we added the sentence like below in line 3 to 5, p16, and line 11 to 14, p16 in Discussion, according to the reviewer's comment.

“However, this study did not show that HA-1077 was equally effective in animal models of bladder cancer developed with the 5637 or UM-UC-3 bladder cancer cell lines.”

“In order to directly address these issues, we should compare the effectiveness of HA-1077 and its vehicle control *in vivo* by developing a mouse model of human bladder cancer in the future.”

The answer to 2

We appreciate the thoughtful constructive suggestions of the reviewer regarding immunohistochemistry. We regret that we did not more carefully cite the immunohistochemistry. So, we corrected the sentence like below in line 13 to 23, p10 in Results, according to the reviewer's comment.

“On immunohistochemical analysis, the cytosolic compartment showed brown staining in most of the cancer cells, indicating high RhoA activity and high ROCK-I and ROCK-II protein levels, while the nuclei showed very weak staining (Figure 2). This staining pattern was identical to that detected in our previous study [21-23]. Most of the cells from both bladder cancer cell lines showed moderate to strong cytoplasmic staining by anti-RhoA antibody, and many tumor cells also showed weak to moderate cytoplasmic staining by anti-ROCK-I and anti-ROCK-II antibodies. HA-1077 reduced the level of reactivity with anti-ROCK-I and anti-ROCK-II antibodies to very weak staining in <20% of tumor cells, while the extent of staining for anti-RhoA antibody was not changed much, but its intensity was reduced to weak or moderate.”

The answer to 3

We appreciate the thoughtful constructive suggestions of the reviewer regarding statistical analysis. We agreed the reviewer’s comment. So, we have performed additional statistical analysis, according to the comment of the reviewer. The biggest change in this revised version is that we examined the difference in the percentage of proliferated, apoptotic or migrated cells at each HA-1077 concentration was compared between cultures with HA-1077 alone and cultures with HA-1077 plus LPA and GGOH. Similarly, in this revised study, we also examined the change of Rho activity and expression of ROCK-I and ROCK-II between cultures with HA-1077 alone and cultures with HA-1077 plus LPA and GGOH. Therefore, our conclusions in this revised manuscript that HA-1077 prevents the proliferation and migration of bladder cancer cells and also induces apoptosis by inhibiting ROCK may be more accurate than our conclusions in the previous version.

So, we corrected the Results like below, according to the reviewer's comment.

“**Inhibition of cell proliferation by HA-1077**

We examined the inhibitory effect of HA-1077 on the *in vitro* growth of human bladder cancer cell lines. Addition of LPA and GGOH increased cell proliferation along
with upregulation of RhoA activity and elevation of expression of ROCK-I and ROCK-II (Figures 4 and 5). Cell proliferation was inhibited by HA-1077 in a dose-dependent manner, both when HA-1077 was added alone and when it was added in combination with LPA and GGOH (Figures 4A and 5A). Expression of ROCK-I and ROCK-II was significantly decreased by HA-1077 in a dose-dependent manner, but RhoA activity was only reduced slightly (Figures 4B-D, and 5B-D).

On the other hand, comparison between cells treated by HA-1077 alone and those treated by HA-1077 in combination with LPA and GGOH revealed that RhoA activity was higher in the latter cells at each HA-1077 concentration (Figures 4B and 5B), while the difference in the expression of ROCK-I and ROCK-II gradually became smaller at higher HA-1077 concentrations (Figures 4C,D and 5C,D).

**Induction of apoptosis by HA-1077**

We examined the effect of HA-1077 on apoptosis of human bladder cancer cells in vitro. Addition of HA-1077 to cultured cells led to marked induction of apoptosis in a dose-dependent manner compared with control cultures, and this effect was seen for both HA-1077 alone and HA-1077 combined with LPA and GGOH (Figures 6A, B). When the difference in the percentage of apoptotic cells at each HA-1077 concentration was compared between cultures with HA-1077 alone and cultures with HA-1077 plus LPA and GGOH, it gradually decreased at higher concentrations of HA-1077 (Figures 6C, D).

**Influence of HA-1077 on LPA-induced cell migration**

We next examined the effect of HA-1077 on the migration of cultured human bladder cancer cells. Addition of LPA and GGOH increased the migration of bladder cancer cells compared with control cultures (Figures 7A, 8A). Cell migration was suppressed by HA-1077 in a dose-dependent manner, both in cultures with HA-1077 alone and in cultures with HA-1077 plus LPA and GGOH (Figures 7A and 8A). At the same time, RhoA activity and the expression of ROCK-I and ROCK-II were all significantly reduced by HA-1077 in a dose-dependent manner (Figures 7B-D and 7B-D). The dose-dependent down-regulation of the expression of these proteins by HA-1077 is likely to occur in parallel to the reduction in the number of migrating cells. Regarding changes of protein expression, the difference of ROCK-I and ROCK-II expression between cultures with HA-1077 alone and cultures with HA-1077 plus LPA and GGOH gradually decreased at higher concentrations of HA-1077 (Figures 7C, D and 8C, D). In contrast, RhoA activity was higher in the latter cultures at each HA-1077 concentration (Figures 7B, 8B).

**Minor essential revisions:**

The answer to 4

We appreciate the thoughtful constructive suggestions of the reviewer. We agreed the reviewer’s comment. We regret that we did not more carefully cite. As the reviewer suggested, we corrected like below in line 1, p7, in Materials and Methods.

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We appreciate the thoughtful constructive suggestions of the reviewer. We agreed the reviewer’s comment. So, we deleted the first ten sentences and the entire third paragraph in Discussion, according to the reviewer's comment.

The answer to 6

We appreciate the thoughtful constructive suggestions of the reviewer. We agreed the reviewer’s comment. So, we deleted the last paragraph in Discussion, according to the reviewer's comment.

The answer to 7

We appreciate the thoughtful constructive suggestions of the reviewer. We agreed the reviewer’s comment. So, we deleted eight references which were included in the sentences of the first ten sentences, the entire third paragraph and last paragraph in Discussion which will be deleted in this revised manuscript (the answer to 5 and 6), and we did not add any additional information in this revised version, according to the reviewer's comment.

Reply to reviewer 2: Dr Ivan Nabi

We appreciate the thoughtful constructive suggestions of the reviewer.

We understand the necessary of investigation of in vivo animal model to explore the roles of HA-1077 in cancer treatment, however, at present, we can not afford instrument and device to run experiment using in vivo animal model. Thus, we added the sentence like below in line 3 to 5, p16, and line 11 to 14, p16 in Discussion, according to the reviewer's comment.

“However, this study did not show that HA-1077 was equally effective in animal models of bladder cancer developed with the 5637 or UM-UC-3 bladder cancer cell lines.”

“In order to directly address these issues, we should compare the effectiveness of HA-1077 and its vehicle control in vivo by developing a mouse model of human bladder cancer in the future.”

Additional points:

The answer to 1

We appreciate the thoughtful constructive suggestions of the reviewer.

We regret that we did not more carefully cite. As the reviewer suggested, we examined phosphorylation of RhoA (RhoA activity) by measuring its GTP-bound active form.

The answer to 2

We appreciate the thoughtful constructive suggestions of the reviewer.
We regret that we did not send suitable figure regarding cell culture. We agreed the reviewer’s comment. So, we removed green background in new Figure 1, according to the reviewer's comment.

The answer to 3

We appreciate the thoughtful constructive suggestions of the reviewer regarding statistical analysis. We agreed the reviewer’s comment. So, we have performed additional statistical analysis, according to the comment of the reviewer. The biggest change in this revised version is that we examined the difference in the percentage of proliferated, apoptotic or migrated cells at each HA-1077 concentration was compared between cultures with HA-1077 alone and cultures with HA-1077 plus LPA and GGOH. Similarly, in this revised study, we also examined the change of Rho activity and expression of ROCK-I and ROCK-II between cultures with HA-1077 alone and cultures with HA-1077 plus LPA and GGOH. Therefore, our conclusions in this revised manuscript that HA-1077 prevents the proliferation and migration of bladder cancer cells and also induces apoptosis by inhibiting ROCK may be more accurate than our conclusions in the previous version.

So, we corrected the Results like below, according to the reviewer's comment.

**Inhibition of cell proliferation by HA-1077**

We examined the inhibitory effect of HA-1077 on the *in vitro* growth of human bladder cancer cell lines. Addition of LPA and GGOH increased cell proliferation along with upregulation of RhoA activity and elevation of expression of ROCK-I and ROCK-II (Figures 4 and 5). Cell proliferation was inhibited by HA-1077 in a dose-dependent manner, both when HA-1077 was added alone and when it was added in combination with LPA and GGOH (Figures 4A and 5A). Expression of ROCK-I and ROCK-II was significantly decreased by HA-1077 in a dose-dependent manner, but RhoA activity was only reduced slightly (Figures 4B-D, and 5B-D).

On the other hand, comparison between cells treated by HA-1077 alone and those treated by HA-1077 in combination with LPA and GGOH revealed that RhoA activity was higher in the latter cells at each HA-1077 concentration (Figures 4B and 5B), while the difference in the expression of ROCK-I and ROCK-II gradually became smaller at higher HA-1077 concentrations (Figures 4C,D and 5C,D).

**Induction of apoptosis by HA-1077**

We examined the effect of HA-1077 on apoptosis of human bladder cancer cells *in vitro*. Addition of HA-1077 to cultured cells led to marked induction of apoptosis in a dose-dependent manner compared with control cultures, and this effect was seen for both HA-1077 alone and HA-1077 combined with LPA and GGOH (Figures 6A, B). When the difference in the percentage of apoptotic cells at each HA-1077 concentration was compared between cultures with HA-1077 alone and cultures with HA-1077 plus LPA and GGOH, it gradually decreased at higher concentrations of HA-1077 (Figures 6C, D).

**Influence of HA-1077 on LPA-induced cell migration**

We next examined the effect of HA-1077 on the migration of cultured human bladder cancer cells. Addition of LPA and GGOH increased the migration of bladder cancer cells compared with control cultures (Figures 7A, 8A). Cell migration was suppressed by HA-1077 in a dose-dependent manner, both in cultures with HA-1077 alone and in
cultures with HA-1077 plus LPA and GGOH (Figures 7A and 8A). At the same time, RhoA activity and the expression of ROCK-I and ROCK-II were all significantly reduced by HA-1077 in a dose-dependent manner (Figures 7B-D and 7B-D). The dose-dependent down-regulation of the expression of these proteins by HA-1077 is likely to occur in parallel to the reduction in the number of migrating cells. Regarding changes of protein expression, the difference of ROCK-I and ROCK-II expression between cultures with HA-1077 alone and cultures with HA-1077 plus LPA and GGOH gradually decreased at higher concentrations of HA-1077 (Figures 7C, D and 8C, D). In contrast, RhoA activity was higher in the latter cultures at each HA-1077 concentration (Figures 7B, 8B). “

Reply to reviewer 3: Dr Martina Schmidt

Major compulsory revisions:

The answer to 1 and 5

We appreciate the thoughtful constructive suggestions of the reviewer regarding statistical analysis. We agreed the reviewer’s comment. So, we have performed additional statistical analysis, according to the comment of the reviewer. The biggest change in this revised version is that we examined the difference in the percentage of proliferated, apoptotic or migrated cells at each HA-1077 concentration was compared between cultures with HA-1077 alone and cultures with HA-1077 plus LPA and GGOH. Similarly, in this revised study, we also examined the change of Rho activity and expression of ROCK-I and ROCK-II between cultures with HA-1077 alone and cultures with HA-1077 plus LPA and GGOH. Therefore, our conclusions in this revised manuscript that HA-1077 prevents the proliferation and migration of bladder cancer cells and also induces apoptosis by inhibiting ROCK may be more accurate than our conclusions in the previous version.

So, we corrected the Results like below, according to the reviewer's comment.

“Inhibition of cell proliferation by HA-1077

We examined the inhibitory effect of HA-1077 on the in vitro growth of human bladder cancer cell lines. Addition of LPA and GGOH increased cell proliferation along with upregulation of RhoA activity and elevation of expression of ROCK-I and ROCK-II (Figures 4 and 5). Cell proliferation was inhibited by HA-1077 in a dose-dependent manner, both when HA-1077 was added alone and when it was added in combination with LPA and GGOH (Figures 4A and 5A). Expression of ROCK-I and ROCK-II was significantly decreased by HA-1077 in a dose-dependent manner, but RhoA activity was only reduced slightly (Figures 4B-D, and 5B-D).

On the other hand, comparison between cells treated by HA-1077 alone and those treated by HA-1077 in combination with LPA and GGOH revealed that RhoA activity was higher in the latter cells at each HA-1077 concentration (Figures 4B and 5B), while the difference in the expression of ROCK-I and ROCK-II gradually became smaller at higher HA-1077 concentrations (Figures 4C,D and 5C,D).

Induction of apoptosis by HA-1077

We examined the effect of HA-1077 on apoptosis of human bladder cancer cells in vitro. Addition of HA-1077 to cultured cells led to marked induction of apoptosis in a
dose-dependent manner compared with control cultures, and this effect was seen for both HA-1077 alone and HA-1077 combined with LPA and GGOH (Figures 6A, B). When the difference in the percentage of apoptotic cells at each HA-1077 concentration was compared between cultures with HA-1077 alone and cultures with HA-1077 plus LPA and GGOH, it gradually decreased at higher concentrations of HA-1077 (Figures 6C, D).

**Influence of HA-1077 on LPA-induced cell migration**

We next examined the effect of HA-1077 on the migration of cultured human bladder cancer cells. Addition of LPA and GGOH increased the migration of bladder cancer cells compared with control cultures (Figures 7A, 8A). Cell migration was suppressed by HA-1077 in a dose-dependent manner, both in cultures with HA-1077 alone and in cultures with HA-1077 plus LPA and GGOH (Figures 7A and 8A). At the same time, RhoA activity and the expression of ROCK-I and ROCK-II were all significantly reduced by HA-1077 in a dose-dependent manner (Figures 7B-D and 7B-D). The dose-dependent down-regulation of the expression of these proteins by HA-1077 is likely to occur in parallel to the reduction in the number of migrating cells. Regarding changes of protein expression, the difference of ROCK-I and ROCK-II expression between cultures with HA-1077 alone and cultures with HA-1077 plus LPA and GGOH gradually decreased at higher concentrations of HA-1077 (Figures 7C, D and 8C, D). In contrast, RhoA activity was higher in the latter cultures at each HA-1077 concentration (Figures 7B, 8B). “

The answer to 2

We appreciate the thoughtful constructive suggestions of the reviewer regarding with positive and negative control in western blotting. We regret that we did not more carefully cite the condition. We stated positive and negative control in our previous manuscript regarding Rac1 activity in upper urinary tract cancer, which was published in BMC Cancer at 2010 [ref. 23]. In the present study, we run experiment of western blotting by same method. So, we added the reference like below in line 10, p9 in Materials and Methods, according to the reviewer's comment.

“Hela cells were used as the positive control as described previously [23]. “

The answer to 3

We appreciate the thoughtful constructive suggestions of the reviewer. We regret that we did not send suitable figure regarding cell culture. We agreed the reviewer’s comment. So, we removed green background in new Figure 1, and added the sentence like below in line 11 to 12, p10 in Results, according to the reviewer's comment.

“Cell proliferation was inhibited by HA-1077 in a dose-dependent manner (Figure 1). “

The answer to 4
We appreciate the thoughtful constructive suggestions of the reviewer. We understand the necessary of investigation of *in vivo* animal model to explore the roles of HA-1077 in cancer treatment, however, at present, we can not afford instrument and device to run experiment using *in vivo* animal model. Both of 5637 and UM-UC-3 bladder cancer cell lines could form subcutaneous tumors quickly in nude mice as well as colonies in a clonogenic assay *in vitro*. Thus, we added the sentence like below in line 3 to 5, p16, and line 11 to 14, p16 in Discussion, according to the reviewer's comment.

“However, this study did not show that HA-1077 was equally effective in animal models of bladder cancer developed with the 5637 or UM-UC-3 bladder cancer cell lines.”

“In order to directly address these issues, we should compare the effectiveness of HA-1077 and its vehicle control *in vivo* by developing a mouse model of human bladder cancer in the future.”

The answer to 6

We appreciate the thoughtful constructive suggestions of the reviewer regarding with statistical analysis. We agreed the reviewer’s comment. We regret that we did not more carefully cite the condition regarding potential mechanisms leading to the reduction of ROCK expression by HA-1077.

So, we added the sentence like below in line 4 to 13, p14 in Discussion, and in line 26, p16 to line 7, p17 in Conclusions, according to the reviewer's comment.

“LPA increases GTP loading, while GGOH activates geranylgeranylation. The mevalonate pathway is required for geranylgeranylation of Rho by GGOH. After Rho has been activated by geranylgeranylation, its downstream effector ROCK is activated when it selectively binds to the active GTP-bound form of Rho. In the present study, addition of LPA and GGOH to cultured cells increased RhoA activity and up-regulated the expression of ROCK-I and ROCK-II, while HA-1077 dramatically suppressed both ROCK-I and ROCK-II dramatically, but did not reduce RhoA activity. These findings indicate that HA-1077 may selectively inhibit bladder cancer cell proliferation and migration via suppression of ROCK, but not by blocking RhoA activity.”

“To explore the underlying molecular mechanisms, we treated bladder cancer cells with HA-1077 and then examined changes of the GTP-bound active form of RhoA and expression of its downstream effector ROCK. Treatment with HA-1077 caused a decrease in the growth and migration of bladder cancer cells, while apoptosis showed a significant increase. Expression of ROCK-I and -II proteins was decreased by exposure of tumor cells to HA-1077, while RhoA activity was not affected. These findings indicate that HA-1077 prevents the proliferation and migration of bladder cancer cells and also induces apoptosis by inhibiting ROCK, suggesting that ROCK may be a molecular target for the treatment of cancer.”