Author's response to reviews

Title: Synergistic effects of acyclovir and 3, 19-isopropylideneandrographolide on herpes simplex virus wild types and drug-resistant strains

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Author's response to reviews: see over
13 January 2015

Dear Editor,

We are extremely grateful to the editor and referees who certainly did a thorough job at reviewing our previous version of the manuscript. We realize that the previous version is unclear. We have now complied with all observations and suggestions and we are convinced that the manuscript is now significantly stronger and hope it is now suitable for publication.

Please find below a point-by-point response (R#) to the raised comments (Comment #) while some changes have been highlighted in the revised manuscript.

We would be very appreciated if you could consider our manuscript for publication in your journal.

Sincerely yours,

Dr. Chamsai Pientong
Response to Reviewer #1: Angel S. Galabov

Comment 1.1 (i) The individual antiviral effects of IPAD are not, unfortunately, markedly selective at all. Selectivity index values of 2.2 - 2.36 (Table 1) characterize these effects as borderline ones, i.e. IPAD could not be considered as an antiviral substance. Consecutively, this substance is not prospective as anti-HSV agent. It could be of interest what will be the in vivo activity of IPAD, in experimental HSV infections in laboratory animals.

R1.1

- We are grateful to the reviewer for the insightful comment.

We agree with your comment that SI value of IPAD is quite low and IPAD could not be considered as an anti-HSV alone. In the previous version of manuscript, we did not point an important result that in the combination assay, IC$_{50}$ of ACV and IPAD were reduced more than 100 and 10 times, respectively when compared with the single drug assay. This result indicated the increased SI value of IPAD from 2 (in the single drug assay) to 20 (in the combination assay). Therefore, IPAD might be considered as an antiviral substance in combination drug treatment for HSV infection. This information has been added in the revised manuscript. Please see highlighted in the chapter “Results” lines 205-208.

In addition, IPAD showed inhibitory effects on both HSV wild types and drug resistant strains and might have the specificity of action different from ACV. In this study, we would like to demonstrate the synergistic effect. IPAD should be useful to combine with anti-HSV agent as ACV to avoid ACV high dose and completely inhibit HSV drug resistant strains. Some compounds such as glucoevatromonoside that inhibited HSV-1 and HSV-2 infections with SI 2.107 and >6.250, respectively was
also shown the synergistic antiviral effects with acyclovir to inhibit HSV-1 and HSV-2 replication by the inhibition of viral proteins synthesis (Bertol JW et al., 2011).

We also agree with your suggestion to perform experiments in laboratory animals. *In vivo* study is our further study. Now, the study is granted and approved by the Animal Ethics Committees. In this present study, we would like to show the potential effect of IPAD from *in vitro* study.

Reference:

Comment 1.2 (ii) In the chapter “Discussion” are missed data on other studies on the substances (e.g. of plant origin) possessing inhibitory effects against HSV ACV resistant strains (e.g. Vilhelmova-Ilieva et al., Antiviral Research 110, 2014, 104).

R1.2
We are grateful for the suggestion regarding the missed data.

We add the following information in the revised manuscript. Please see highlighted in the chapter “Discussion” lines 242-244 and 254-256. This reference has been added as well.

“The inhibitory effects of plant-derived polyphenolic compounds, castalagin, vescalagin and grandinin (C-glucosidic ellagitannins containing nonahydroxyterphenoyl), on the replication of ACV-resistant strains of HSV-1 and HSV-2 were shown in MDBK cells”

“and that the combination of ellagitannin(s) and ACV exhibited a much stronger synergistic effect against ACV-resistant HSV-1 compared to ACV-resistant HSV-2”
Comment 1.3 (iii) Ganciclovir (p. 3, line 56) is not an anti-HSV drug; it was proved as an anti-HCMV agent and is recommended for clinical use with this indication.

R1.3
We are thankful to the reviewer for the wonderful observation. It was our mistake. In the revised manuscript, Ganciclovir has been deleted.

Response to Reviewer #2: Laura B Talarico

Major compulsory revisions
Comment 2.1) I strongly recommend reviewing the English grammar and style of the manuscript. The manuscript is not well-written and it has a tremendous amount of syntax errors (lines 41-44, 80-81, 83-86, 100-101, 113-114, 120-122, 123-125, 163-165, 172-173, 209-211, 211-215, 216-223, 248-251, to mention some). It becomes difficult to understand what the authors mean to say in many sentences.

R2.1) We are grateful for the suggestion. The manuscript has been revised by authors and the revised manuscript has been edited by a native English-speaking scientist as described in the Acknowledge.

Comment 2.2) The manuscript has also important concept mistakes, such as the use of units (uM) in selectivity indexes (SI) (line 173 and Table 1). SI is the ratio CC50/IC50 and does not have units.

R2.2) We are embarrassed by the mistake of using units. The units have been deleted.
Comment 2.3) The viral entry assay that the authors used is in fact a virucidal assay, since there is an incubation between the virus and the compound before adding the mixture to the cells. The entry assay should be performed separately to evaluate if there is an effect on virus binding (adsorption of virus to Vero cells at 4°C for 1 h in the presence of compound) and internalization (infection of Vero cells at 37°C for 1 h in the presence of compound) to the cells.

R2.3) We would like to thank you for your suggestion and are sorry that we did not clearly describe about the experiment. For the viral entry assay, we performed firstly for virucidal assay and then investigated for effect on virus binding and internalization as described below. The results were not significantly different. We use new words “pre-entry step of infection” in the revised manuscript.

For virus binding or attachment assay as described by Su et al., 2008, the Vero cells were grown in a 96-well culture plates and pre-chilled at 4 °C for 1 h. The cell monolayer was then infected with HSV-1 (~50 PFU/well) in the presence or absence of compound at indicated concentrations and incubated at 4 °C for 1h. The infected cell monolayer was then washed three times with cold PBS and cultured at 37°C for 72h for CPE reduction assay.

The penetration (internalization) assay was performed as described by Cheng et al., 2005. The Vero cells were grown in 96-well culture plates and pre-chilled at 4 °C for 1 h. The cell monolayer was then infected with HSV-1 (50 PFU/well) and incubated at 4 °C for another 2 h to allow virus attachment to the cell monolayer. After that, various concentrations of tested compound were added. The infected cell monolayer was then incubated at 37 °C for 10 min to maximize virus penetration. PBS at pH 3 was added for 1 min to inactivate unpenetrated virus, and PBS at pH 11 was then added immediately to neutralize the acidic PBS. The neutral PBS was removed and the cell monolayer was cultured at 37°C for 72h for CPE reduction assay.
From these experiments, IPAD showed little inhibitory effects as shown in Figure A and B. This manuscript described only the result of the virucidal assay.

![Figure A. virus binding or attachment assay](image)

![Figure B. internalization or penetration assay](image)
References:

Comment 2.4) The MOI used in the plaque reduction assays do not make sense. If the assays were performed in 24-well plates (this is not stated in the manuscript), in each well there are approximately 2.5x10^5 cells, so an MOI of 0.01 means that the authors are able to count 2.5x10^3 PFU in a well.

R2.4) - We are thankful to the reviewer for the wonderful observation.
It was our error that we did not describe clearly. We have been added a new detail in the revised manuscript. Please see the highlight in the chapter “Methods” Lines 122-123 and 132-133. In this study, the CPE reduction assay was performed on 96-well culture plates that were seeded with 50 µl of Vero cells at 2x10^5 cells/ml (10^4 cells/well). When 0.01 MOI of HSV was used, the virus control showed approximately 80-90 foci of CPE under an inverted microscope.

Comment 2.5) The selectivity indexes of IPAD against HSV strains are very low (around 2), which means that this compound is not a good antiviral agent against HSV. A desirable SI for an antiviral agent should be >10. The authors performed the antiviral assays at a concentration of 22.5 uM (where they see 50-60% inhibition) which is very near the CC50 (39.71 uM), so it is difficult to distinguish antiviral from cytotoxic effects. This important issue makes the compound a very poor antiviral agent. The authors do not mention this important limitation of the study.

R2.5) - We are grateful to the reviewer for the insightful comment.
We agree with your comment that SI value of IPAD is quite low and IPAD could not be considered as an anti HSV alone.

In the previous version of manuscript, we did not describe some important results. In a single drug assay, the lowest concentration of IPAD which completely inhibited CPE formation by all HSV strains at post-entry step was 20.50 µM. In combination assay, IC\textsubscript{50} of ACV and IPAD were reduced more than 100 and 10 times, respectively when compared with the single drug assay. This result indicated the decreased SI value of IPAD from 2 (in the single drug assay) to 20 (in the combination assay). Therefore, IPAD might be considered as an antiviral substance in combination drug treatment for HSV infection. This information has been added in the revised manuscript. Please see highlighted in the chapter “Result” lines 207-210.

In addition, IPAD showed inhibitory effects on both HSV wild types and drug resistant strains and might have the specificity of action different from ACV. In this study, we would like to demonstrate the synergistic effect. IPAD should be useful to combine with anti-HSV agent as ACV to avoid ACV high dose and completely inhibit HSV drug resistant strains. Some compounds such as glucoevatromonoside that inhibited HSV-1 and HSV-2 infections with SI 2.107 and >6.250, respectively was also shown the synergistic antiviral effects with acyclovir to inhibit HSV-1 and HSV-2 replication by the inhibition of viral proteins synthesis (Bertol JW et al.,2011).

We also agree with your suggestion to perform experiments in laboratory animals. \textit{In vivo} study is our further study. Now, the study is granted and approved by the Animal Ethics Committees. In this present study, we would like to show the potential effect of IPAD from \textit{in vitro} study.

Reference:
Comment 2.6) A good antiviral agent should have a selectivity index > 10, so that the antiviral assays could be performed at a concentration near the IC90 (so as to have 90% inhibition of viral plaques).

R2.6) We are thankful to the reviewer for the suggestion. In this study, we calculated SI (selectivity index) by using the common formula (SI = CC50/IC50). We also try to calculate as your suggestion and the result showed that SI values are around 2.

Comment 2.7) For the antiviral assays against drug-resistant strains of HSV, the authors used a concentration of ACV of 2220 uM which is higher than the CC50 (>1000 uM). The authors should evaluate that the range of concentrations that they are using for the antiviral assays are non-cytotoxic.

R2.7) We did not clearly describe. In cytotoxicity assay, ACV was diluted from 50 to 6,400 µM. The result showed that all concentrations of ACV were not toxic to Vero cells. This information has been added in the chapter “Methods” and “Results.” Please see the highlighted Lines 111-112 and 170-171.

Comment 2.8) The CC50 of the combination of IPAD and ACV was not determined and should be included in the manuscript.

R2.8) The CC50 of the combination of IPAD and ACV was determined and has been included in the revised manuscript.

Minor Essential Revisions

Comment 2.9) The references should be revised, since specific studies are cited as the source of general information regarding HSV epidemiology and pathogenesis (line 54).
We are thankful to the reviewer for insightful observation about wrong references. Now we have revised the reference citation.

Comment 2.10) The authors do not mention the strain of HSV type 2 that they used in the study.

In this study, we used HSV-2 clinical isolate. We have added it in the revised manuscript. Please the highlighted in the chapter ‘Methods’ line 93.

Comment 2.11) The authors do not mention the staining method used to count plaques (crystal violet).

We observed CPE and counted the foci of CPE under an inverted microscope. This information has been added in the revised manuscript.

Comment 2.12) The method used for the calculation of the potential synergistic effect should be described in more detail and clearly.

The synergistic effect of ACV and IPAD was calculated by using a combination index (CI) as described previously (Chou T-C: Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 2006, 58:621-681). CI = [(D)(1)/(D(2)]) + [(D)(2)/(D(2)] where: (D(2)) = (D)(2) alone that inhibited a system x%, (D(2)) = (D)(2) alone that inhibited a system x% and (D)(1) + (D)(2) in combination also inhibited x%. A CI value of 1 indicates an additive effect; CI < 1 indicates a synergistic effect, and CI > 1 indicates an antagonistic effect. Please see the highlighted in the chapter “Methods” lines 161-164.

Comment 2.13) In Figure 2, the legend for ACV2 is missing.

The legend for ACV2 has been added in Figure 2.
Comment 2.14) In Figure 3, the authors mention that IPAD reduced the expression of viral protein, but there is gD expression of drug-resistant HSV strains determined by western-blot.

R2.14)
We are thankful to the reviewer for insightful observation about the wrong legend.
There are wrong labelling of Lane 2 and Lane 3 in Figure 3.
Lane 2: HSV infected cells treated with IPAD (20.50 µM)
Lane 3: HSV infected cells treated with ACV (20.20 µM)
We have edited already.