Author's response to reviews

Title: A new anti-glioma therapy, AG119: Pre-clinical assessment in a mouse GL261 glioma model

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Author's response to reviews: see over
Response to Reviewers

We would like to thank the Reviewers for their insightful comments and suggestions that have improved the quality of our manuscript. We have responded to each comment individually below. Hopefully we have addressed all the issues to the Reviewers’ satisfaction.

Reviewer 1

Major concerns:
1. Methods section: the vehicle for dissolving AG119 should be provided.

Now included in the Methods section, which reads as follows:
“AG119 was dissolved in 5% N-methylpyrrolidine (Pharmasolve; Sigma-Aldrich), 5% solutol-15 (BASF, Bern, Switzerland) in sterile normal saline. TMZ was dissolved in 5% DMSO and 5% solutol-15 in sterile saline. Antibody therapies (anti-c-Met (B-2): sc-8057; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and anti-VEGF (anti-mouse VEGF-A; Biolegend Inc., San Diego, CA) were prepared in sterile saline.”

2. Figure 2A, which day did the tumor volume data generate from? Can the tumor volume data be presented as a function of treatment/implantation days? Figure 2A shows AG119 had smaller tumor size than TMZ treated ones, which did not correlate with the animal survival data presented in Figure 1 (TMZ treated mice live longer), any explanation for that?

MRI was not necessarily done on the last day of survival due to scheduling time on the scanner. Also we had two mice that died from complications associated with anesthesia for the AG119 treatment group, which may not appropriately represent the survival data for this group. We have included a statement in the Discussion regarding this issue, which reads: “Also of note, the survival data included anesthesia-related deaths for the AG119 treatment group which may not properly reflect the actual survival times for this treatment group, and should be repeated in future studies.”

3. Figure 2Ei, 2Eii, and 2Eiii represent TMZ-treated mice at 29-31 days, are those images acquired from the same mouse? Why 2Eii (day 30) has smaller tumor size than 2Ei (day 29) and 2Eiii (day 31)?

The times have been corrected, and a statement has been added in the Figure legend as follows: “Each image is obtained from different mice in each treatment group.”

4. Figure 2Fi and 2Fii represent AG119-treated mice at 28-29 days, what about 2Fiii? No description in the figure legend.

The days following implantation range is for all animals in group F. We have updated Fig. 2 to include more representative images from groups E (TMZ) and F (AG119), and included individual times on each image.

5. Figure 3 Ci/Cii vs. Figure 3Di/Dii: will tumor size affect the measurement of perfusion?

Perfusion rates were obtained for total tumor tissue in appropriate image slices. Perfusion rates are affected by treatment response, which can affect tumor volumes.
Minor concerns:
1. Line 130, the MDA-MB-435 cells were not true breast cancer cell line, they were found to be melanoma cell lines (Ellison G, Klinowska T, Westwood RF, Docter E, French T, Fox JC. Further evidence to support the melanocytic origin of MDA-MB-435. Mol Pathol. 2002 Oct;55(5):294-9.).

The MDA-MB-435 line was a kind gift of Dr. Janet Price at MD Anderson Cancer Center in the mid-1990s and was shown to be free of melanoma cross-contamination (Chambers, A. F. (2009). MDA-MB-435 and M14 Cell Lines: Identical but not M14 Melanoma? Cancer Research, 69(13), 5292–5293. doi:10.1158/0008-5472.CAN-09-1528). We added this reference and adjusted the text in the section in question.

Reviewer 2

There are some discrepancies that should be corrected and some details that it would be beneficial to add:

A) The methods say 5-6 mice per group where figure legends indicate an n of 7 for AG119.

*Changed in Methods to read “5-7 mice per group”.*

B) It would be good to document how antibody therapies are delivered (IP?).

*This has now been added to the Methods. They were administered i.v. via a tail-vein catheter.*

C) Line 158 states that “euthanized 1-2 days prior to expected disease-initiated deaths”. It would be good to expand upon the criteria that were used to determine that the mice were moribund since survival analysis is used as one measure of therapy efficacy.

*We added the criteria used to determine when to euthanize the mice, as follows:*

“All animals were humanely euthanized (CO₂ asphyxiation) when they met tumor burden criteria (tumors ≥ 150 mm³) and/or showed signs of illness, weight loss, poor body condition, porphyria, hypoactivity, restlessness, aggressiveness, ataxia, shallow, rapid and/or labored breathing, cachexia, failure to respond to stimuli, lack of inquisitiveness, vocalization, seizures, hunched posture and ruffled fur. In some circumstances a few animals (n=2) died due to anesthesia complications, but were included in the survival data.”

D) For viability assays, from the methods it is unclear if concentrations of vehicle (DMSO) were controlled for in experiments to determine the IC50 of TMZ and AG119. Although the amounts of DMSO added to cells for AG119 studies are calculated to be minimal; for TMZ studies it seems like high potentially detrimental concentrations of DMSO would be present in cells treated with higher amounts of drug. If not controlled for, this could lead to an under-estimation of IC50 values.

*Temozolomide (TMZ) is most soluble in DMSO (39 mg/ml = 51.5 mM), thus the experiment was limited by the solubility of the drug. In a previous experiment, we had tested the effect of DMSO on the same cells and found that 1% DMSO (500 µM TMZ) resulted in less than a 5% decrease in viability compared to untreated cells.*
E) For animal studies were non-treat animals injected with vehicle or saline as a control? If not could this influence results?

*We clarified in the Methods that the untreated controls were injected with the vehicle, which reads as follows:* “Control untreated tumor-bearing mice received the same solvent as for those that were treated with AG119 (vehicle control).”

In a couple of instances it appears that the conclusions may somewhat overstate the data.

A) In line 205 it is stated that “AG119 compared well against other anti-glioma therapies including anti-VEGF and anti-c-Met antibody therapies, or TMZ, regarding both survival and tumor volumes.” Given that AG119 was inferior to TMZ in survival, this statement should be updated to reflect that fact.

*We have reworded the above statement in the Results section, and specifically indicated how AG119 compared to TMZ.*

B) In line 217 it would be clearer to phrase it that T98G and U251 cells had similar sensitivities to AG119 (As opposed to saying “not resistant”).

*This statement was added to the results section:* “It was also found that TMZ-resistant T98G cells were as sensitive to AG119 as the TMZ-sensitive U251 cells (Fig. 4; IC50 comparison).”

C) In line 241 it is stated that “In this work we showed that AG119 is also not subject to MGMT mediated resistance, as is the case with TMZ.” This is based on the fact that both T98Gs and U251s have a similar IC50 value for AG119. However to nail this down definitively, the authors would need to show that T98Gs and T98Gs with MGMT knock-down are equally sensitive to AG119 (and a similar experiment for TMZ over-expression in U251s). This reviewer does not feel that these experiments are necessarily required for publication of this paper, but feels that the discussion should be modified to indicate the limitations of the experiment.

*This statement was added to the discussion section:* “This work suggests that AG119 is also not subject to MGMT mediated resistance, as is the case with TMZ. A study to further explore this idea would be a comparison of AG119 sensitivity in parental U251 cells as compared to T98G cells with MGMT knocked out.”

In reference 13, AG119 appears to be referenced to as 3·HCl. It would be good to add that detail to the introduction.

*We included the reference to 3•HCl for AG119 as previously referred to in reference 13.*

There are discrepancies that should be corrected and some suggested changes that would make it easier for a reader to follow the paper.

A) In line 58 it might be clearer instead of “have not improved overall survival” to say “have not substantially improved overall survival” to indicate the fact that studies have found that temozolomide added to radiotherapy extended survival by 2 months compared to radiotherapy alone. A similar change is suggested in line 114 as well.

*Changed as suggested.*
B) In line 68 should the p value <.05 really be <.001 as indicated in Figure 1?  
*Changed.*

C) In line 70 should the “and” before anti-c-Met be deleted?  
*The sentence has been clarified.*

D) Figure legend 1 should be updated to include the anti-c-Met and anti-VEGF groups that are shown on the graph.  
*Updated as suggested.*

E) The Results/Discussion begins rather abruptly. It would also help the flow of the article if the various results/discussion were split into new paragraphs with a sub-heading and a sentence or two added to briefly summarize the model employed and the experimental design.  
*We split the Results and Discussion sections, and re-organized the Results and added introductory statements as suggested.*

F) Overall, the writing about perfusion rates is hard to follow. It seems in certain instances that the word “decreased” is used when it is actually referring to the ability of tested drugs to increase perfusion rates in tumors compared to no treatment.  
*We have clarified the perfusion sub-section in the Results, and in the Fig. 3 legend, as suggested.*

**Reviewer 3**  
Comments:  
In order to compare the efficacy of AG119 with other standard therapies it is important to provide the information regarding those are drugs. For example: how did they choose the concentration of the Abs? For example, Marchant and coll (PNAS, 2013, p 110(32): E2987–E2996) showed that antibodies against human c-Met (onartuzamab) had different effect at different concentrations: 1 and 3.75 mg/kg delayed tumor growth, whereas doses of #7.5 mg/kg drove tumor regression in animal model. Please provide information and citations for anti-c-MET and anti-VEGF.  
*We have added a reference for the antibody therapies in the Methods, and have included the following statement in the Discussion:  
“It should be noted that antibody therapies in this study were not optimized, i.e. doses used elicited therapeutic responses, but did not necessarily induce tumor regression [27]. Also of note, the survival data included anesthesia-related deaths for the AG119 treatment group which may not properly reflect the actual survival times for this treatment group, and should be repeated in future studies.” Reference 27 is the suggested Merchant et al, 2013 article.*

What’s the vehicle in which AG119 is resuspended? Why the control animals did not receive the vehicle?
We have updated the Methods section to include the suspension media for each treatment group. See comment 1 for Reviewer 1 above.

FIG1: Kaplan Meier: In the result section the authors said: “Percent survival of GL261 HGG-bearing mice treated with AG119 was significantly higher (p<0.05) compared to untreated tumors, as depicted in Fig. 1. TMZ, however was found to have a significant increased percent survival when compared to AG119 (p<0.01)” However in the figure legends they showed the statistical analysis for tumor volume. Those data clearly showed that TMZ has a stronger therapeutic efficacy than AG119 and that AG119 has the same efficacy than anti-c-Met and anti-VEGF.

We have corrected the discrepancy noted above in the relevant sections.

FIG 2:
Based on the Kaplan Meier plot, anti-VEGF, anti-c-Met and AG119 has the same therapeutic efficacy against GL261. All animals treated with these different agents died by day 28. However the MRI imaging in fig.2 clearly showed strong tumor regression or absence of tumor (Fii) in animals treated with AG119. Why did the animal died? Animals that received TMZ only partially respond to the treatment and show strong tumor growth. Why these MRI imaging do not correlate with the survival plot.

MRI was not necessarily done on the last day of survival, due to scheduling on the scanner, which may account for some misalignment of the data. We also had two mice in the AG119 treatment group that died due to anesthesia complications, and their deaths were included in the survival data. Please also see the response to comment 2 for Reviewer 1 above.

Is AG119 extremely toxic for these animals? According to their previous paper 3 HCL (now called AG119) is not toxic. How do they know if AG119 can cross the BBB? Clearly TMZ has a stronger therapeutic effect and AG119 does not improve life expectancy.

AG119 is not toxic as was previously reported by our group and included in the manuscript. In pre-clinical models for gliomas we have previously shown that the blood-tumor-barriers in these tumor-bearing mice are very leaky, and so we do not anticipate a problem with AG119 reaching its’ target. Appropriate references and a statement has been added to the Discussion, to read as follows:

“It has also been previously reported that the blood-tumor-barrier in GL261 gliomas are quite “leaky” which allows the penetration of various therapeutic compounds [27], as well as molecular targeting agents which we have reported on [28].”