**Author's response to reviews**

**Title:** In vivo glioblastoma growth is reduced by apyrase in a rat glioma model

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**Author's response to reviews:**

August 1st, 2006

Dr. Sally Simpson
Assistant Editor

Dear Dr. Simpson

We are submitting the revised version of our manuscript 2026859046986483.
The new title of the manuscript is: "In vivo glioblastoma growth is reduced by apyrase in a rat glioma model"
Bellow you can find:
1) the answers to the reviewers questions and,
2) the list of point-by-point of the main modifications done in the manuscript.
Thank you very much for the opportunity to review the manuscript.

Sincerely yours,

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ANSWERS TO REVIEWERS

Reviewer: Pier Andrea Borea
Two minor essential points should be addressed:

1- Work in the literature already ties the purinergic system to cancer. Moreover, one report in Biochem Pharmacol shows adenosine as a key element in glioblastoma development (Merighi S., et al., Biochem Pharmacol. 2006;72:19-31.).

We are aware of Merighi's work and agree that apyrase injection may increase adenosine around the tumor, but this does not seem to be increasing the expression of VEGF, since we found a decrease (Figure 5C of the manuscript). This apparent inconsistency may be due to the differences of in vivo versus in vitro conditions.

We tried to address this problem by treating C6 glioma cells in culture with apyrase, and preliminary data indicated that the expression of VEGF mRNA did not change. A more direct strategy of testing adenosine and other purinergic agonists is under way, but these results are still too preliminary to be included in the present manuscript.

We agree that Merighi's work is important to be cited. Considering that the whole discussion about adenosine involvement in VEGF expression in this model would require a deeper analysis, we only included a short addition (pg 15) and the reference in the discussion, as shown below:

"As mentioned above, angiogenesis is an extremely important process for sustained tumor growth. Studies have demonstrated that C6 and human gliomas cell lines secrete VEGF [6,35, 36].

2- The authors show that the presence of an ATP-depleting enzyme at the moment of glioma implantation causes a significant decrease in the growth, angiogenesis and proliferation index. The exact mechanisms to explain these observations remain under evaluation, however, the participation of ATP and the ecto-nucleotidases may be associated with the development of this type of brain tumor in an in vivo glioma model. A small scheme could be added as a last figure in order to indicate what the main take home conclusions of the work are.

We are thankful for the suggestion, but we think that the figure would be more appropriate for a review paper (in preparation), which would also include data from other papers of our group and other groups.

Reviewer: Cinzia Volonte
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) The data not shown concerning the uptake of glutamate in C6 glioma cells (at the beginning of the Results section) should be omitted, not having direct relevance to the work.

We agree and took it out.

2) Figure 2 and Figure 3 should be combined, since Fig. 2 (Temozolomide sample) is a positive control for the new results depicted in Fig. 3.

We agree and combined the two figures.

3) Conversely, the lack of effect by apyrase on in vitro growth/survival of C6 cells should be presented, providing evidence that apyrase is not toxic to these cells. Do the authors further know if apyrase interferes with cell cycle, cell duplication time in vitro? This could have important mechanistic implications on the results presented.

We have tested the effect of apyrase on cell proliferation treating C6 cultures with the same proportion used in the co-injection experiments (2U apyrase/106 cells). The results obtained showed that the enzyme was not toxic to the cells but instead stimulated by around 40% the cell number after 24h of treatment. This stimulatory effect could be due to the production of adenosine from the endogenous secreted ATP. On the other hand, the experiments in vivo with co-injection of apyrase reduced the glioma growth after 20 days.
This apparent difference may be due to other mechanisms involved in vivo versus in vitro conditions. Moreover, considering that the injection of apyrase was done only at the moment of implantation, probably the effect of ATP depletion by apyrase is more important for the implantation and initial growth of the tumor, than the potential effect of adenosine at this moment. We did not evaluate if apyrase interferes with cell cycle, cell duplication time in vitro, but we consider the suggestion very relevant to further investigate the mechanistic implications of the purinergic system in the glioma implantation.

We agree with the relevance of the data showing that apyrase was not toxic to cells, but instead of including these results in a new figure we think that to cite the results in text would be more appropriated. If the reviewer considers extremely relevant to show the data in a figure, we can do this. The following phrase (Results, pg 11 and 12) was changed in order to clarify the idea about the non-toxic effect of apyrase:

"The effect of apyrase (2U apyrase/106 cells) on C6 cell proliferation in culture showed that the enzyme was not toxic to the cells but instead stimulated by around 40% the cell number after 24h of treatment, indicating that apyrase did not reduce the viability of the injected cells".


This excellent review came out after we submitted the manuscript. This was now added.

5) A little editing should eliminate typing or English mistakes.

Done.

Discretionary Revisions (which the author can choose to ignore)

6) What happens to glioblastoma growth in the in vivo model if extracellular ATP is added instead of apyrase?

ATP was shown to be cytostatic in several types of cancer, but we believe that in our in vivo model, the gliomas are favored by ATP, which promotes the tumor growth and the normal brain tissue death. Besides, considering the high toxicity of extracellular ATP for neurons, this experiment would be technically challenging, therefore requiring a tight control of concentration, which is not easily attainable in vivo.

7) Details should be provided directly on Table 1 on how coagulative necrosis, intratumoral hemorrhage, lymphocytic infiltration, and so on were measured.

Details were added to the legend of table 1.

Reviewer: Rafal Czajkowski

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

The results presented in the manuscript figures seem reasonable, significant and very striking. However, it has not been explained how a single, acute injection of an active enzyme would provide sufficient apyrase activity for prolonged period (20 days). It is generally accepted that the activity of extracellular proteases is relatively high in tumors (the authors even mention this fact in the manuscript, p. 4, line 12 and subsequent citation). Therefore it is very unlikely that the enzymatic activity of the injected apyrase would persist for more than a few hours.

We agree that apyrase will most probably loose activity over a period of 20 days. But, we think that this does not invalidate our conclusions. Our suggestion is that the degradation of ATP by apyrase will negatively influence implantation and initial growth of the tumor cells. Perhaps this was not clearly stated in the discussion. Therefore we added the following (pg 14):

"Our experiments demonstrate that the co-injection of apyrase significantly diminished the growth of implanted gliomas in rats after twenty days of tumor induction. Considering that the injection was done only at the moment of injection, probably the effect of
ATP depletion by apyrase is important for the implantation and at the initial growth of the tumor. We think that this could be of therapeutic interest, since the application of apyrase in the surgical resection cavity could be helpful in reducing the initial growth of invaded tumor cells.

The observed phenomenon could be explained by a more reasonable mechanism. After harvesting, C6 cells are collected and handled in DMEM without growth factors until the injection. Under such stressful conditions (lack of both adhesion and serum), ATP might act in an auto/paracrine manner as the growth factor that prevents cell death. Therefore at the very moment of the inoculation, a significant fraction of the glioma cells treated with apyrase might be already unable to form the tumor. This possibility is fully justified by the previous results obtained by the authors (p. 5 lines 3-8) and other groups.

We share the preoccupations about the viability of the cells. We would like to clarify that all cells were maintained in DMEM with serum. In fact, revising the material and methods section, we discovered a mistake when describing the conditions in which the cells were maintained. We meant DMEM + serum at the end of the 1st phrase of the glioma implantation part (pg. 6). Therefore we changed this phrase to: "Rat C6 glioma cells at around 70% confluency were trypsinized, washed once in DMEM/5% serum, spun down and resuspended in the same medium."

In addition, the cells were not incubated with apyrase prior to injection. Only at the very moment of injection, the C6 cells were resuspended in a medium containing apyrase (2U apyrase/106 cells).

Thanks for pointing out this mistake.

The control experiment performed by the authors (p. 12, lines 15-17) does not reproduce the experimental conditions because the glioma cells are not detached from the surface. These issues have to be addressed experimentally in order to make the results reliable.

As suggested by the reviewer, now we have done another control in order to reproduce the experimental conditions. The C6 cells were prepared in the same way as we used for injection in rat brain. The cells were detached from the surface and counted to obtain the proportion of injected cells. Then we divided in two groups: without apyrase (control group) and the C6 cells were resuspended in a medium containing 2U apyrase/106 cells apyrase (apyrase group). The cell viability was evaluated by Trypan blue exclusion and MTT assays (briefly described below). It was observed that the cells treated with apyrase were more than 90% viable when compared to the control cells, indicating that the cells might be able to form the tumor. We added a brief description of this control in material and methods (pg 6) and a new paragraph to give this information (pg. 12).

**MTT cell viability assay**

The suspended cells were transferred to a 96-well plate and 20 µl of the CellTiter 96AQueous One Solution Reagent was added and incubated for 30 and 60 min. The absorbance was read by an ELISA plate reader at 490 nm.

First, the amount of viable glioma cells in the brain after injection needs to be confirmed. The suggested time points are 24 and 48 h post injection. The standard tests for apoptosis and proliferation would certainly be convincing.

We thoroughly thought about these questions, but all kinds of tests we tried to do did not produce reliable quantitative data.

1. We tried to look at animals after 10 days of implantation and we could not identify a clear tumor. A histological analysis 24 or 48h after implantation would require a marker of gliomas and we are unaware of any unmistakable marker. We also tried to transduce gliomas with EGFP, but these cells had a slower growth rate and did not produce reliable tumors, therefore this strategy was also dropped.
2. Plating the region around the injection would also have cells from the circulation, such as lymphocytes and macrophages and also cells from the CNS, such as astrocytes and microglia. An unmistakable identification of the gliomas would therefore require multiple passages, at which time the experiment would have lost most of its quantitative nature.

Also, a simple (but slightly less convincing) method to perform this control would be to re-plate the cells handled exactly as for injection and to compare their survival rates after 24 and 48 h in DMEM/FCS.

This control was done. The C6 cells handled exactly as for injection were re-plated and their survival rates after 24 and 48h in DMEM/FCS were evaluated by Trypan blue exclusion. As for the evaluation of cell viability just after the apyrase treatment (described above), the cells treated with apyrase and re-plated were
more than 90% viable when compared to the control cells, indicating that the cells might be able to form the tumor. These results, added to the manuscript, indicate that the effect of apyrase is not probably related with the initial viability of the injected cells but rather with the subsequent tumor implantation and growth.

Second, the activity of apyrase in the tumor tissue has to be monitored during the experiment. At least several crucial time points (1 hour, 1 day, 7 days and 20 days) are necessary. After collection of the tissue, the presence of the intact protein (western blot) or enzymatic activity (hopefully this is possible in such a small sample) should be detected. If the results of the mentioned control experiments are positive (i.e. the initial number of viable cells is indeed lower, and/or the apyrase activity is decaying), the whole issue should be readdressed.

Again, we totally agree with the concerns raised by the referee, but unfortunately all these experiments are technically very challenging and probably only slightly quantitative. Our intention in the present work was to investigate the participation of ATP at the initial moment of glioma implantation using an in vivo model and an enzyme that is known to be able to deplete ATP. Moreover, as stated above, we think that the apyrase activity is very probably not maintained over the period of 20 days, but which does not invalidate the conclusions of this work.

There are several ways to tackle the problem. A molecular approach: to enhance the expression of the apyrase in glioma cells (preferably in an inducible manner) and compare tumors with induced and non-induced enzyme expression.

We are thankful for the suggestion. This approach is being undertaken, but difficulties in producing C6 stably expressing apyrase have delayed this part of a work in preparation. Despite this, we still believe that the observations reported in this manuscript stand by itself and are worth while publishing.

A surgical approach: to provide constant supply of apyrase to the forming tumor, for example by using the osmotic pump, starting no earlier than 48 h after inoculation.

Our concern with this approach is that the presence of a microdialysis probe would lesion the injection site, significantly changing the feature of the tumor growth. It would also not ensure that apyrase reached all parts of the tumor. We also don't think that this approach would be clinically relevant. Therefore the molecular approach seems more reasonable, and we are moving towards this aim.

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

The manuscript is well written, but the title could be more specific. Since ATP is studied only indirectly, mentioning apyrase in the title would be more appropriate.

We accept the reviewer suggestion and we propose the new title below:

"IN VIVO GLIOBLASTOMA GROWTH IS REDUCED BY APYRASE IN A RAT GLIOMA MODEL"

The introduction and discussion are generally well balanced, with one exception. On page 5, par. 3 (the hypothesis), lines 15-16, the authors hypothesized that ATP might act by inducing cell death of surrounding normal tissue and creating more space for growing tumor. This particular possibility is not tested in the subsequent sections (no test for apoptosis in surrounding tissue, just within the tumor itself) and therefore should be moved to the Discussion.

We agree with the reviewer and we have re-written the Introduction and Discussion in order to present our hypothesis and discuss the results more adequately. In the page 5 (third paragraph), the sentences: "In glioma cell lines, nucleotides and nucleosides induce proliferation [16], and ATP can mediate death in dissociated primary cerebellar granules or striatal neurons and in hippocampal organotypic cultures [20]" and "The indirect autocrine nature of this process should be noted; the tumor induces, through the death of normal cells, the release of more ATP that stimulates its own multiplication and, as a consequence, the cell death of other cells opening space to be occupied by tumor cells and liberating more ATP to continue the
LIST OF MODIFICATIONS DONE

1. TITLE
As suggested by the reviewer we changed the title to:

IN VIVO GLIOBLASTOMA GROWTH IS REDUCED BY APYRASE IN A RAT GLIOMA MODEL

2. BACKGROUND
In the page 5 the sentences: "In glioma cell lines, nucleotides and nucleosides induce proliferation [16], and ATP can mediate death in dissociated primary cerebellar granules or striatal neurons and in hippocampal organotypic cultures [20]" and "The indirect autocrine nature of this process should be noted; the tumor induces, through the death of normal cells, the release of more ATP that stimulates its own multiplication and, as a consequence, the cell death of other cells opening space to be occupied by tumor cells and liberating more ATP to continue the invasive processed" were moved to the discussion (pg 14).

3. METHODS
- In the page 6 we included the concentration of serum used with DMEM.
- In the page 6 we added a brief description of the control of cell viability:

"Cell viability was evaluated by trypan blue exclusion and MTT assays".

4. RESULTS
- The paragraph concerning to the uptake of glutamate in C6 glioma cells was omitted;
- The Figure 2 and Figure 3 were combined and the respective legend was adapted;
- In the pages 11 and 12, the following paragraph was included:

"The effect of apyrase (2U apyrase/106 cells) on C6 cell proliferation in culture showed that the enzyme was not toxic to the cells but instead, induced an increase by around 40% of the cell number after 24h of treatment. Additionally, the C6 cells were detached from the surface handled exactly as for the injection (with or without apyrase) and were subsequently re-plated in DMEM/FCS for 24 and 48h. Cell viability was analyzed by trypan blue exclusion and MTT assays immediately after the cell preparation for injection and after 24 and 48h of culture. The cells presented more than 90% viability when compared to the control cells in all conditions tested, indicating that the effect of apyrase is not probably on the initial viability of the injected cells but rather on the subsequent tumor implantation and growth."

5. DISCUSSION
- The following phrase in page 14 was changed:

"Our experiments demonstrate that the co-injection of apyrase significantly diminished the growth of implanted gliomas in rats after twenty days of tumor induction. Considering that the injection was done only at the moment of injection, probably the effect of ATP depletion by apyrase is important for the implantation and at the initial growth of the tumor. We think that this could be of therapeutic interest, since the application of apyrase in the surgical resection cavity could be helpful in reducing the initial growth of invaded tumor cells."

- A short addition and the reference 36 were included in page 15, as shown below:

"As mentioned above, angiogenesis is an extremely important process for sustained tumor growth. Studies
have demonstrated that C6 and human gliomas cells lines secrete VEGF [6, 35, 36].

6. REFERENCES

Two new references were included:


7. LEGENDS

- Details were provided on the legend of Table 1;
- The Figure 2 and Figure 3 were combined and the respective legend was adapted;

8. GENERAL:
An English editing was done throughout the manuscript.