Author's response to reviews

Title: ARG098, a novel anti-human Fas antibody, shows strong cytotoxic effects, suppresses synovial hyperplasia, and prevents cartilage destruction in rheumatoid arthritis.

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Author's response to reviews: see over
Proffesor John Isaacs,
Ms Nina Titmus,
The BioMed Central Editorial Team,

Dear Proffesor Isaacs,

RE: MS: 4752772533600702 entitled "ARG098, a novel anti-human Fas antibody, shows strong cytotoxic effects, suppresses synovial hyperplasia, and prevents cartilage destruction in rheumatoid arthritis"

Thank you for your mail on 7\textsuperscript{th} May 2010 and for the referees’ comments concerning the above manuscript. I am pleased to learn that Musculoskeletal Disorders is interested, in principal, in publishing our manuscript with the extensively edit. We are attaching a revised manuscript and a letter to the referees. I hope that the revised manuscript is now acceptable for publication in Musculoskeletal Disorders.

Sincerely yours,

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The major revised places

<table>
<thead>
<tr>
<th>Place of the original</th>
<th>Original</th>
<th>Revised</th>
<th>Reasons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title</td>
<td>ARG098, a novel anti-human Fas antibody, shows strong cytotoxic effects, suppresses synovial hyperplasia, and prevents cartilage destruction in rheumatoid arthritis.</td>
<td>ARG098, a novel anti-human Fas antibody, suppresses synovial hyperplasia and prevents cartilage destruction in a severe combined immunodeficient-HuRAg mouse model</td>
<td>Following the suggestion of the reviewer 2, we changed the title.</td>
</tr>
<tr>
<td>Abstract</td>
<td>-</td>
<td>We have reduced the abstract length and modified the strong statement.</td>
<td>Following the suggestion of the reviewer 2.</td>
</tr>
<tr>
<td>Background</td>
<td>-</td>
<td>The second and the most of sixth paragraph of original manuscript were deleted.</td>
<td>Following the suggestions of the reviewers 1 and 2.</td>
</tr>
<tr>
<td>Method “Human chondrocyte culture” and “Detection of apoptosis by DNA nick end labeling and histopathological analysis”</td>
<td>-</td>
<td>We have simplyfied the method.</td>
<td>To avoid the redundancy of the manuscript.</td>
</tr>
<tr>
<td>Method “Lactate dehydrogenase (LDH) release assay”</td>
<td>-</td>
<td>The section was deleted.</td>
<td>Following the suggestion of the reviewer 2, some figures were deleted from the manuscript. LDH data was deleted.</td>
</tr>
<tr>
<td>Method “Preparation of SCID-HuRAg mice” and “Preparation of SCID-HuRAg and”</td>
<td>-</td>
<td>These two sections were combined.</td>
<td>To avoid the redundancy of the manuscript.</td>
</tr>
<tr>
<td>Results “ARG098 binding ability and cytotoxic potency in Jurkat cells” and Figure 1</td>
<td>-</td>
<td>The section and Figure 1 were deleted.</td>
<td>Following the suggestion of the reviewer 2, this figure was deleted in the manuscript and the manuscript volume was reduced.</td>
</tr>
<tr>
<td>Results “Induction of apoptosis in RA synoviocytes” and Figure 2E</td>
<td>-</td>
<td>Figure 2E was deleted and the section was modified.</td>
<td>Following the suggestion of the reviewer 2, this figure was deleted in the manuscript and the manuscript volume was reduced.</td>
</tr>
<tr>
<td>Results “Induction of apoptosis in cytokine-treated RA synoviocytes”</td>
<td>-</td>
<td>The efficacy comparison between ARG098, CH-11, and 7C11 was added in this section.</td>
<td>Following the suggestion of the reviewer 1, the efficacy comparison data between anti-Fas antibodies was added.</td>
</tr>
<tr>
<td>Results “ARG098 does not induce apoptosis in human hepatocytes” and Figure 5</td>
<td>-</td>
<td>The section and figure 5 were deleted.</td>
<td>Following the suggestion of the reviewer 2, this figure was deleted in the manuscript and the manuscript volume was reduced.</td>
</tr>
<tr>
<td>Results “ARG098 binding ability and cytotoxic influence in human chondrocytes”</td>
<td>-</td>
<td>Figure 6 C and D have been combined to become Figure 4C.</td>
<td>The figures were combined in one graph to simplify the results.</td>
</tr>
<tr>
<td>Discussion</td>
<td>-</td>
<td>We have rewrote the section. Some information concerning the synoviocytes sensitivity were added to</td>
<td>Following the suggestions of the reviewers 1 and 2, some important reports were added in</td>
</tr>
<tr>
<td>Figures and Figure legends.</td>
<td>-</td>
<td>Figure 1, figure 2E, Figure 5, Figure 8E were deleted.</td>
<td>Following the suggestion of the reviewer 2, these figures were deleted in the manuscript and the manuscript volume was reduced.</td>
</tr>
<tr>
<td>Figures</td>
<td>-</td>
<td>Type fonts and sizes for labeling of figures and figure axes have been standardized throughout the manuscript, and given some textual explanations within the figures.</td>
<td>Following the suggestions of the reviewer 1, figures were modified to be helpful for readers in quickly interpreting data.</td>
</tr>
</tbody>
</table>
We found the reviewer’s comments very helpful and have revised the manuscript accordingly.

**Major Compulsory Revisions:**

1) The authors report the superior effect of ARG098 in inducing apoptosis in Jurkat cells and RA FLS compared to historical attempts. To make this claim, the authors need to show ARG098 side by side with another anti-Fas antibody such as CH-11. In particular, they should examine dose responses of another Fas antibody in Jurkat cells, and find concentrations of this antibody and ARG098 which induce comparable levels of apoptosis in Jurkat. Then, the two antibodies should be compared at these concentrations in RA FLS. Only then can the authors claim any superiority of the ARG098 compound.

As we have mentioned in the manuscript, a previous study showed that the anti-Fas IgM antibody clone CH-11 (CH-11) at 25 ng/mL induced cell death in about 60% of Jurkat cells [1], and 50 ng/mL induced cell death in almost all Jurkat cells [2]. Another agonistic anti-Fas monoclonal antibody, the mouse IgM clone 7C11, was reported to induce apoptosis in about 80% of Jurkat cells at concentrations of 10 ng/mL and above [3].

Based on these results, we performed several experiments by comparing the efficacies of ARG098, CH-11, and 7C11 in RA synoviocytes preincubated with TNF-α. ARG098 at a concentration of 100 ng/mL induced cytotoxicity in more than 90% (93.7 ± 1.29%, mean ± S.E.M.) of RA synoviocytes in this condition, whereas clone 7C11 at 100 ng/mL induced cytotoxicity in 65.5 ± 4.38% of RA synoviocytes. Clone CH-11 at 5000 ng/mL induced cytotoxicity in 9.56 ± 3.54% of RA synoviocytes. From these results, ARG098 is expected to be superior to the other antibodies. However, we have revised the manuscript to indicate that the superiority is suggested and not conclusive: in the Abstract we have mentioned, “These results suggest that ARG098 is a novel and efficacious for RA treatment,” and in the Conclusion section, “Taken together, these results suggest that ARG098 will be an efficacious RA therapy.”

**Reference**

2) While the authors attribute the historical lack of efficacy of anti-Fas antibodies on RA FLS to, among other things, their need for multimerization, a very good recent study by Pundt and colleagues (Arthritis Res Ther, 2009, 11/1/R16) demonstrated that RA FLS susceptibility to FasL-induced death was exquisitely dependent upon the proliferation rate of cells in vitro, as well as cell cycling status and culture confluence. The authors need to take into consideration that their positive results with ARG098 are secondary to tissue culture conditions –eg., there is no evidence in the methods section that they serum-starve their FLS or achieve cell cycle arrest.

The culturing conditions for the RA synoviocytes have an impact on their susceptibility to the Fas signal. In this study, all the RA synoviocytes used were within the fifth passage, which means that they retained their primary cell characteristics. Medium changes and cell passages were carefully performed to avoid confluent growth in the culture dishes. The medium used for culturing the RA synoviocytes included 10% FBS. The RA synoviocytes used in our experiments were not synchronized with the cell cycle, and the cells had mixed cell cycling and proliferation statuses. In our culture conditions, ARG098 induced cell death in about 60% of the RA synoviocytes; the remaining 40% of the synoviocytes may be in a condition, phase, or cell cycle that is resistant to Fas-mediated cell death [1]. Therefore, following the suggestion of the reviewer, we have revised the Discussion section to include a consideration of cell cycling status.

Reference


3) The authors ignore a number of important manuscripts published on this topic such as effects of PI3 kinase signaling in RA FLS resistance to Fas-dependent apoptosis from the group of Pope, and others. Additionally, they fail to examine the effect of their antibody on synovial macrophages, an important synovial population reported to be resistant.

It has been previously reported that the PI3K/Akt-1 pathway is activated in RA joints and that this pathway induces apoptosis-inhibitory factors [1]. Therefore, following the suggestion of the reviewer, the Discussion section has been revised to include a consideration of the PI3K/Akt-1 pathway.
RA synoviocytes prepared using the methods used in our study are heteromorphous and can be differentiated into three types: dendritic cells, macrophage cells, and fibroblast-like cells [2]. We believe that ARG098 affects all the three types of synovial cells and that these effects are evaluated by our assay system. However, as indicated by the reviewer, RA synoviocytes resistant to ARG098 treatment may include synovial macrophages and we consider this as a reason for the resistance to several anti-Fas antibodies shown by the population of synovial cells used in each experiment.

Reference

4) Both introduction and discussion sections are unnecessarily long, a result of inadequate organization and internal redundancies – both sections could be shortened by 33-50%.

Following the suggestions of the reviewer, some paragraphs have been deleted in the manuscript, and the manuscript length (Introduction and Discussion sections) has been reduced.

5) Extreme care should be given to grammatical errors appearing throughout the manuscript.

Following the suggestion of the reviewer, the revised manuscript has been edited by a native English speaker.

Minor Essential Revisions:
1) Type fonts and sizes for labeling of figures and figure axes should be standardized throughout manuscript.

Following the suggestion of the reviewer, the labeling of figures has been revised.

2) Figures 1, 2 and 4-6 are very similar in format of data presentation – giving some textual explanation within the figure as to the cell type studied (beyond doing this in the figure legend) would be helpful for readers in quickly interpreting data.
Following the suggestion of the reviewer, some explanations have been added in the figures.
Referee 2:

We found the reviewer’s suggestions very helpful and have revised the manuscript accordingly.

Major points:
- The title is something misleading because the work is not performed in either a true experimental model of RA not in RA patients. The authors should discuss why is not possible to study this drug in a mouse transgenic for human-Fas (Fas-KO mouse exist).

As indicated by the reviewer, this study was not performed in RA patients. We have revised the title to “ARG098, a novel anti-human Fas antibody, suppresses synovial hyperplasia and prevents cartilage destruction in a severe combined immunodeficient HuRAg mouse model.”

Mice transgenic for human Fas are also of interest for evaluating antibody efficacy. We used a SCID HuRAg mouse model because this model has been established to mimic human RA affected joints. Several clinical studies have shown good clinical efficacy of the agents that were successful in the SCID HuRAg mouse model [1-4], especially monoclonal antibodies highly specific for human molecules [1, 2]. The SCID-HuRAg mouse model is one of the best models available and we therefore used it to evaluate ARG098.

Reference

- From these results it is not clear the mechanism of action of ARG098 because the authors do not detect a clear increase in apoptosis. Probably they should use control positive apoptotic cells induced by Fas-ligand in order to confirm if they can detect apoptosis or they have problems with apoptotic signaling in their cells. Similarly, the lack of effects of this drugs on
PBMC, hepatocytes and condrocytes/cartilage is intriguing and remains to be well explained and more experiment are needed before to conclude that this drug has no toxic effects on these cells. Indeed the lack of toxic effects of this drug should be demonstrate at the systemic level (animal model).

ARG098-induced cell death was evaluated with annexin-V/PI staining according to the apoptosis measuring method of Aubry et al [1]. ARG098 at a concentration of 100 ng/mL induced annexin-V-positive cells in RA synoviocytes within 6 h of treatment. This result shows that ARG098 induced apoptosis in vitro.

As mentioned by the reviewer, the lack of toxic effects of ARG098 on PBMCs, hepatocytes, and chondrocytes/cartilage is intriguing. Other safety studies performed on ARG098 in human tissue slices in vitro and cross-reactive animals in vivo did not show any toxicity toward PBMCs, hepatocytes, or chondrocytes/cartilage (unpublished data). We believe that ARG098 may have no systemic toxic effects on these cells or organs, at least in the range of concentrations for intra-articular administration.

Reference

- As ARG098 is a IgM monoclonal antibody and IgM can activate the complement system, it would be interesting to role out that activation of complement is implicated in the cytotoxic effects of this drug.

ARG098 clearly induced apoptosis in vitro and we believe that part of the suppression of synovial hyperplasia by ARG098 is based on its induction of apoptosis. On the other hand, as indicated by the reviewer, it has also been reported that IgM can activate the complement system. Therefore, ARG098 may also reduce hyperplasia through a complement-dependent cell death mechanism. Following the suggestion of the reviewer, the Discussion section has been revised to include the point regarding complement system activation.

- Abstract should be largely reduced and strong statements on the anti-RA effect of this drug should be modified or eliminated.

Following the suggestion of the reviewer, we have decreased the length of the abstract and modified
the strong statements.

- Background is too long and contains paragraphs on treatment of RA which no reflect the current approach to the therapy of RA (prosthesis and synovectomy are every time less performed in RA management). This introduction needs be re-written more concisely and more focused on the relevance of the target and in the rationale of the study (including possible undesirable effects of anti-Fas).

Following the suggestion of the reviewer, the Background section has been rewritten, and some sections have been deleted and modified taking the rationale of the study, including the possible undesirable effects of the anti-Fas antibody, into consideration.

- Discussion is also is very long and little focused in the real translational relevance of their findings. It should include limitations of the study as well as future research needed to propose this drug as potential therapy for RA (Surprisingly we read in Conclusion that this drug is now in Phase I/II clinical trials).

Following the suggestion of the reviewer, the Discussion section has been rewritten, and some sections have been deleted and modified.

Minor points:
- Methods and Results should be reduced or some part of them (some figures) could be included as supplemental material).

Following the suggestion of the reviewer, some figures have been deleted from the manuscript and the length of the manuscript has been decreased.

- There is redundancy and a lot of spelling mistakes throughout all the manuscript and therefore it should be revised in deep by an expert English writer.

Following the suggestion of the reviewer, the revised manuscript has been edited by a native English speaker.

- In pag. 20, last paragraph, the authors state "synovium infiltrating lymphocytes.... consisting
of T cells, B cells, macrophages and neutrophils and these lymphocytes..." Please modify.

The sentence indicated has been deleted when the Discussion section was rewritten.