Title: Effects of cytarabine on activation of human T cells cytarabine has concentration-dependent effects that are modulated both by valproic acid and all-trans retinoic acid

Authors:

Elisabeth Ersvær Dr. (elisabeth.ersver@hib.no)
Annette K Brenner Dr. (Annette.Brenner@k2.uib.no)
Kristin Vetås (kristin.vetas@gmail.com)
Håkon Reikvam Dr. (Hakon.Reikvam@k2.uib.no)
Øystein Bruserud Prof. (oystein.bruserud@helse-bergen.no)

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Author’s response to reviews: see over
We hereby submit a Revised Version of the previously submitted article entitled: "Effects of cytarabine on activation of human T cells - cytarabine has concentration-dependent effects that are modulated both by valproic acid and all-trans retinoic acid" by Elisabeth Ersvær, Annette K Brenner, Kristin Vetås, Håkon Reikvam and Øystein Bruserud. We hope our Revised Version can be considered for publication in your journal.

We are grateful for the reviewers' comments and have done our best to address all the concerns raised by the reviewers. A detailed point-to-point reply to the referees' comments is given below. In addition, a revised manuscript is attached to this document. All substantive changes are highlighted in yellow.

We hope that you will find our manuscript improved by this revision and we hope that you will now find it suitable for publication in BMC Pharmacology and Toxicology.

Yours sincerely,

Elisabeth Ersvær
Øystein Bruserud
Major Compulsory Revisions

1. Lines 187-188: “ATRA and valproic acid did not cause any statistically significant alteration of T cell viability and proliferation.” However, Figure 2B shows a statistically significant decrease in viability after treatment with 1000 µM valproic acid. Please edit the text to more accurately describe the data.

_The text has been rewritten to a more accurately description of the data (see lines 208-210)._

2. For Figure 2B, should the control sample be at 100%? Please explain how the parent gate was set.

_For all control samples investigated there was a minor cell population that did not undergo proliferation after only four days of culture. These cells had an overlap in Cell Trace Violet MFI equivalent to those control samples with cells that were not activated to undergo proliferation (no stimulation, see Figure 2A). Therefore none of the control samples (activated T cells) showed 100% proliferation; this can be seen by the column bar graph presenting mean with standard deviation (dark grey column with error bars)._ 

3. Lines 213-215: The authors state that “Proliferation and viability was not altered for cultures containing ATRA 1 µM, valproic acid (500 and 1000 µM) and cytarabine (0.01, 0.35, 1.0 and 44 µM”. However, in Figure 2 the data indicates that some of the triple combinations resulted in significantly decreased proliferation. The authors should consider revising their statement.

_We apologize for this mistake. We have now revised this section (see the chapter lines 223-229)._ 

4. The discussion is predominantly a repetition of the results. A substantial revision of the discussion section is required.

_We have revised the Discussion extensively, and we have now included more detailed comments on (i) the possible clinical importance of autologous antileukemic T cell reactivity; (ii) clinical toxicity during AML stabilizing treatment; (iii) indirect effects on other immunocompetent cells caused by altered release of soluble mediators during T cell activation; and (iv) different effects on various T cell subsets. The original parts have been shortened, but we have not shortened the section describing the various doses of cytarabine used in AML treatment. We think that is important to give a relatively detailed explanation of the scientific and clinical background for our selection of cytarabine concentrations in our present study. We hope this is acceptable._

5. Minor Essential Revisions

Some typographical errors need to be fixed:

1. Line 99: a parenthesis is missing or there is a parenthesis that should not be there.
2. Line 105: change “dissolved” to “suspended”.
3. Line 176: change “drug-containg” to “drug-containing”.
4. Line 184: Did you mean CD28?
5. Line 185: Should “100 µM “be “1000 µM”?
7. Line 327: a parenthesis is missing or there is a parenthesis that should not be there.

_The above mentioned typographical errors have been corrected._
Reviewer: Sheryl Flanagan

Major Revisions

1) The authors identified the clinical question of what if any immunomodulatory effects cytarabine in combination with ATRA/valproic acid has on peripheral T lymphocytes and presumably how these effects may contribute to leukocyte recovery after treatment induced deficits. However, the authors’ conclusive findings as stated in the abstract “immunomodulatory effects of low-dose cytarabine are strengthened by ATRA and valproic acid”, lacks specificity, i.e. what effects are strengthened? Are the effects immunosuppressive? What are the clinical implications? Similarly, the first paragraph of the discussion section ends with a sentence that indicates that results of the present study show that low-toxicity chemo will effect T cell system….but how? This is a declarative sentence that lacks detail, rendering it lacking in information.

*We have now rewritten the conclusion in the abstract and the specific effects are given. Due to the overall length of the abstract we find it difficult to discuss possible clinical implications, but this is now discussed more in detail in the revised Discussion section (see below). The overall length of the abstract has not been increased. We hope our solution is acceptable.*

*We have also added a more precise statement to the first chapter of the Discussion section (lines 328-331).*

*Possible clinical implications are now discussed more in detail in the revised Discussion section, especially in the chapter lines 371-404.*

2) Related to point 1) above, a statement of the clinical problem or significance of the work is missing from the abstract. I was able to read between the lines and surmise what the problem being addressed or what the critical questions were because I had first read the authors’ cover letter. A one sentence addition to highlight the problem being addressed or why the work is important or helps to inform the field would add relevance to the abstract and put the study into context of the field.

*One sentence addition to highlight the significance of the work has been included in the first part of the abstract.*

3) The Discussion section overall lacks discussion of how the results inform the field, or interpretation of the results. At times, the Discussion section reads more like Results. What inferences if any can be made from these results? Where do the authors see this work going? How does/could this new information impact this field? I was searching for a discussion of relevancy or significance. While the Conclusion section gives some hint of these points, this section could be expanded, or more conclusive/further interpretation statements could be made within the paragraphs of the Discussion section. Also, the Conclusion section is the first time we read of Treg cells and other background literature that may be related to this present study? This seems to come out of the blue. Including some of this literature in the Background section would help the reader to better put the present study results within the context of what is currently known in the field. Given the background and experience of the authors, an expansion of their interpretations of the data would provide valuable context to how the study results have furthered the field.

*The discussion has been revised (see major comment 4 made by the other reviewer).*

4) For the AML patients used in this study, were these patients receiving low dose cytarabine treatment? Chemo-naïve? If receiving treatment, for how long? The M and M section doesn’t give
details. Also, I am assuming the effect of low dose cytarabine on AML cell viability/proliferative capacity wasn’t already known? If not, this should be stated. If the authors included the work with AML cells to highlight differences between T cells and AML cells, then what do the results mean? Line 371 of the Discussion section makes mention of the differential antiproliferative effects of cytarabine on the two cell types, but not what that may mean clinically.

We have added new information to the material and methods section. Our strategy for recruitment of consecutive patients has been described in detail previously and this reference is now included (lines 175-177).

We included AML cells to our study to compare the in vitro susceptibility of leukemic and normal to various doses of cytarabine; both cell types were then cultured under highly standardized and optimal in vitro conditions. Our proliferation results suggest that there is a therapeutic window with regard to the concentrations with antileukemic effects versus the concentration needed for effects on T cells. This is now commented in the discussion section (lines 361-364).

5) Line 206: viability results of combination of araC and valproic acid are misstated, or do not match the data as presented in Figure 1. Text states, …..the only exception being cytarabine 0.35um that caused a minor reduction of T cell viability when tested alone but not when tested in combination with VA at 1000 um. This statement is true to the figure but what is missing is that the data in the figure indicate that 0.35 um araC in combination with 500 um valproic acid, DOES have a significant effect on viability.

We apologize for this mistake. We have revised this sentence (see lines 209 – 210 and 227-228)

6) Line 240, I think it should read, …..not in the presence of ATRA alone, otherwise the end of this sentence contradicts the first part of the sentence as it is presently written.

We agree and have corrected the sentence (see line 261).

Minor Revisions:
Some grammatical errors/typographical errors need to be addressed. I have listed some below.
1) Line 66: This observation suggests…..
2) Line 72: Remove comma between low-toxicity AML-stabilizing chemotherapy…..
Line 72: One alternative….this word choice suggests alternative to the low toxicity AML-stabilizing chemotherapy described in the prior statement although I believe the authors mean to describe in detail the low-toxicity AML-stabilizing chemotherapy. Would be less confusing if the authors said something like…..one such low-toxicity AML-stabilizing chemotherapy is…….
3) Line 96: drugs were thawed on the same day they were used in experiments…..
4) Line 155: should read either, “the effect of low dose cytarabine was….. Or, the effect of low doses of cytarabine were…..
5) Line 205: awkward sentence….change to….with valproic acid at both 100 um and 500 um.
6) Line 250: combination before supernatant (doesn’t need to be pluralized)
7) Line 310: awkward sentence….the triple combination of……is used to treat AML when…..(in what situations is this triple combination the preferred choice for treatment)
8) Line 311: VA …..has been investigated as an anticancer agent in several clinical studies for the treatment of AML (should be specific here in terms of what cancer),……ATRA….and [eventually] …..I don’t believe eventually fits here. Perhaps, in addition to, or also with….
9) Line 357: …..and then have immunomodulatory effects
All the above mentioned typographical errors have been corrected.

Discretionary Revisions
1) Some of the text is repetitive and perhaps doesn’t need to be included. i.e. the author states numerous times, in M and M, each results section, and figure legends, that the PBMCs were activated by……this detail only needs to be included in the M and M, and perhaps figure legends (even there the reader can be directed to the M and M for details). Removing some of these repetitive sections would leave more room to discuss results.

We have removed some of these repetitions in the text as suggested by the reviewer.