Author's response to reviews

Title: Microarray Analysis Reveals Genetic Pathways Modulated by Tipifarnib in Acute Myeloid Leukemia

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August 12, 2004

Dear Sir/Madam

RE: MS: 2352098793604143

We are submitting revisions to the manuscript entitled “Microarray Analysis Reveals Genetic Pathways Modulated by Tipifarnib in Acute Myeloid Leukemia” for consideration for publication as a research article in *BMC Cancer*.

Below are our comments to each of the reviewer’s specific questions.

**Jason Gotlib**

**Major Compulsory Revisions**

**Q1.** P6-7. The text states that “Seventy-two of these genes were affected in patient samples (p<.05, FDR <.03) and were, therefore considered to be significantly regulated by tipifarnib.” Later, on page8, it is indicated that 1016 genes were significantly changed during farnesyl transferase inhibition in vivo. Please address this inconsistency.

A1. The different numbers originate from the results of different analyses including i) the cell lines (1198 genes were regulated in at least one of the three cell lines), ii) the patient samples (1016 genes were regulated in the two patients), and iii) their intersection (180 genes). 72 of these 180 genes mapped to genetic networks as defined by the pathway analysis tool Ingenuity. We clarified the results at the bottom of p8.

**Q2.** Page 8: Broadly speaking, what proportion of the 1198 genes cited to be significantly regulated in at least one of the cell lines were actually significantly regulated in 2 or 3 of the cell lines instead of just one of the cell lines? If a gene was upregulated (or downregulated) in one cell line, was it consistently upregulated (or downregulated) in the other cell lines?

A2. We have included a document of supplementary data to address this question. On average, approximately 30% of genes regulated in any cell line were also regulated in either one of the other two cell lines. This number is higher than that expected by chance alone as determined by a hypergeometric distribution statistic (p < 0.01). The directions of the expression changes in the cell lines were less consistent than those seen in the
patient samples (see Fig 4). The same observation was made on the set of 1198 genes (supplementary Fig 1A). For example a gene could be regulated differently depending upon the cell line and the day of treatment. This is discussed on p10.

Q3. Similarly, for the 1016 genes found to be significantly changed in the leukemia cells from patients treated with tiparnib, was there a persistent upregulation (or downregulation from days 8 to 22) for these genes, or was there substantial variability in genes being upregulated to downregulated (or vice versa) from time point to time point?
A3. In contrast to the cell lines, the gene expression changes seen in the patient leukemic cells were very consistent. If a gene was up-regulated on day 8 it remained up throughout the 3 week time course (and vice versa). This was true for the vast majority of the 1016 genes regulated in vivo (supplementary Fig 1B).

Q4. K-ras is not among one of the genes evaluated by RT-PCR. Given that it is the only one that is significantly regulated (down-regulated) on the microarray, RT-PCR data should be provided for this gene.
A4. As the reviewer alludes to, of the 72 unique genes that were found to be significantly regulated in both the cell line and patient samples k-ras was the only gene present which is a direct target of farnesyltransferase transferase inhibition. However, it was not the only gene that was significantly down-regulated. We have shown that 23 genes are consistently down-regulated (Fig 4). We did provide quantitative RT-PCR data for a set of 9 genes. Of these adipin and vimentin were from the list of 72 significantly regulated genes. While it would be of interest to profile the expression of k-ras we do not have any patient material left to perform this additional RT-PCR. We are currently screening additional samples from an independent phase 2 clinical study. We could perform the suggested k-ras RT-PCR experiments on those independent samples if this data is deemed essential for warranting publication.

Q5. Was the gene for geranylgeranyltransferase on the microarray, or analyzed by RT-PCR? It would be interesting to evaluate whether this is transcriptionally upregulated with FTI treatment.
A5. Rab geranylgeranyl transferase was present on our microarrays. While it was not regulated in the cell lines it was down-regulated approximately 1.5- fold in the patient samples. However, since it did not meet our selection criteria (being regulated in both cell lines and patients) we have not included this in our discussion.

Q6. P9. I believe the reader requires more information about how the Ingenuity Pathway Analysis Tool creates the networks. For example, what defines the limit about the number of genes or the number of associated pathways that will comprise one network? I raise this point because there are 5 associated pathways in network 1 (which also gives the greatest score (24), whereas there are only 3 associated pathways in networks 2-5.
A6. We have added the following description on p9 under “Identification of genetic networks affected by tipifarnib” to clarify how the Ingenuity Pathway Analysis tool functions: “To this end we carried out pathway analysis on the above 180 genes using the
Ingenuity Pathway Analysis (IPA) tool. Seventy-nine (72 unique) of these 180 genes mapped to genetic networks as defined by the IPA tool. These networks describe functional relationships between gene products based on known interactions in the literature. The tool then associates these networks with known biological pathways.” The IPA tool can include up to 35 genes in each network. The pathways that are listed in Table 4 are representative of the most significantly associated biological pathways as defined by the IPA tool.

Q7. Are the 5 networks shown in Table 3 already generated by the Ingenuity Pathway Tool, or are the networks “built” by the authors by entering into the software associated pathways of interest? For example, for network 1, did the authors have to enter “Immunity, inflammation, apoptosis, cell death, and adhesion,” or is this a network pre-defined by the program? If it is the latter, then it would give more reassurance to the reader that the genes found to be significantly regulated on the microarray fell more nicely into pre-established pathways generated by the computer.

A7. As indicated in A6, the five networks shown in Table 3 were generated by the IPA tool. The biological pathways associated with these networks were also identified by the IPA tool. The biological pathways are pre-defined by the IPA tool based on the literature.

Q8. P. 11, Under “Investigation of apoptosis,” line 8, it is stated that there was a maximum of 23% apoptotic cells at day 5. In looking at the DMSO control, there is approximately 8% apoptosis of cells at day 5. Are these differences in apoptosis (8% vs. 23%) statistically significant? For Figure 6B, are the differences in Annexin V and PI staining (37% vs. 14%) statistically significant?

A8. Figure 6B is an exemplar of the apoptosis assay. To answer the reviewers question we have performed a t-test on the apoptosis data. Apoptosis is increased significantly in both the 100 nM (p = 0.027) and 1 uM (p = 0.032) concentrations of tipifarnib compared to control cells. We have added this to the text on p 11.

Minor Essential Revisions

Q1. Avoid the use of patient initials RH and BS throughout the text, tables and figures. Instead use “patient 1” and “patient 2”

A1. Throughout the manuscript we have replaced patient initials BS and RH with “Patient A and “Patient B”, respectively.

Q2. P 3. Background, 3rd paragraph: It states that RhoE, a second farnesylated small GTPase, is constitutively activated… What specific disease context is this referring to?

A2. RhoE is constitutively active in normal tissues. Since this is not related to specific disease state we have removed this statement from p3.

Q3. P. 10, line 4. Please clarify your use of the term “clustered distally”
A3. “Clustered distally” means that the expression profiles are very different. We have modified the text on p10 line 5 to clarify this.

Q4. P. 11, Under “Investigation of apoptosis,” line 8, change Fig. 4 to Fig 6.
A4. We have changed Fig 4 to Fig. 6 on p11, line 8.

Q5. P. 11, Under “Investigation of apoptosis,” line 8, change Fig. 4 to Fig 6.
A5. We have changed “affects” to “effects” on p10 line 13.

Q6. For reference 7, if what is shown is an abstract, consider using instead the updated publication from Blood, May 1, 2004.
A6. We have updated this reference to the May 1st publication in Blood.

Q7. Reference 9 is the abstract which pre-dated the Blood publication listed in reference number 8. I recommend deleting reference 9 unless it contains information not provided in reference 8.
A7. We have removed reference 9 since it is outdated by reference 8.

Q8. Table 1: Please note if the AML cases were originally de novo or secondary (arising from prior MDS).
A8. Table 1 has been modified to indicate that both patients were diagnosed with de novo AML.

Discretionary Revisions

1. P2. Abstract, line 3: we have removed the comma between FTI and tipifarnib and have added (Zarnestra™, R115777).
2. P2 Abstract, line 3: we have changed “response” to “responses”.
3. P3 Background, line 1: we have changed “investigative therapeutic compound” to “investigative agent”.
4. P3 Background, line 1: we have deleted the word “leading”.
5. P3 Background, line 4: we have changed “is a required process” to “is required for”.
6. P3 Background, line 4: we have deleted the word “therefore”.
7. P3 Background, 2nd para: we have changed the first sentence to “In hematological cancers, tipifarnib has shown significant inhibition…”
8. P3 Background, 2nd para: we have changed the line 5 to “…response rate in patients with refractory or relapsed…”
9. P3 Background, 3rd para, line 1: we have changed “studies on tipifarnib” to “studies with tipifarnib”.
10. P3 Background, 3rd para: we have changed the last two sentences to read “The regulation of these effectors can lead to the modulation of signaling pathways involving cell growth and proliferation, and apoptosis. Thus, FTIs may have complex inhibitory effects on a number of cellular events”.
11. P4 Background, 2nd para, line 8: we have modified this sentence to “…clinical evaluation of the compound’s safety and efficacy in humans.”
12. P4 Background, 2nd para, line 11: we have deleted “the FTI” before tipifarnib.
13. P9 Results and Discussion, 2nd para, line 10: a semi-colon was added “…post-translationally; however, it…”
14. P9 under “Identification of genetic networks affected by tipifarnib”: We clarified the last sentence in the 1st paragraph to “The study by Kamasani et al also found cell cycle pathways were repressed and immunity and cell adhesion pathways were activated by FTI treatment.”
15. P12, line 2: we have deleted “the” in “promise in the hematological malignancies”.

Gilles Favre

Minor Essential Revisions

Q1. The time selected for RNA analysis in cell lines must be clearly defined in page 8.
A1. The time points selected for RNA analysis has been clarified by adding the sentence “Samples for RNA analysis were harvested daily from duplicate cell cultures” to paragraph 2 on p8.

Q2. There are 2 table 3
A2. The Table numbers have been corrected.

We believe we have addressed all of the reviewer’s concerns. Thank you for considering our manuscript and I look forward to your reply.

Yours sincerely,

Dr. Mitch Raponi