Author's response to reviews

Title: Role of Aldo-keto Reductases and Other Doxorubicin Pharmacokinetic Genes in Doxorubicin Resistance, DNA binding, and Subcellular Localization

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Dear Editor, *BMC Cancer*

We were pleased to hear from you that you are interested in publishing our recent manuscript “Role of Aldo-keto Reductases and Other Doxorubicin Pharmacokinetic Genes in Doxorubicin Resistance, DNA binding, and Subcellular Localization”, providing we address the comments of your external reviewers. We thank your reviewers for their informed review and we have extensively revised our manuscript to address their comments and concerns. We have provided a final copy of the revised manuscript in the current submission as well as a marked copy, where changes from the original version of the manuscript can be easily identified. We believe that the revised manuscript is much stronger than our original submission as a result of your review.

Below is an itemized list of the issues raised by your reviewers and how we have modified the manuscript accordingly:

**Review by Elaina Collie-Duguid:**

*a) Identifying the novel aspects of the current manuscript:* Dr. Collie-Duguid is correct that we published a study in 2009 entitled “Induction of 1C aldo ketoreductases and other dose-dependent genes upon acquisition of anthracycline resistance” in *Pharmacogenetics and Genomics*. Consequently, while she appreciates the novel insights into doxorubicin resistance mechanisms presented in our current manuscript, she asked that we make it very clear what findings were observed in our previous study and what novel findings are being highlighted in the current manuscript. In short, the previous study profiled the expression of only 1720 genes as MCF-7 breast tumour cells acquired required resistance to the chemotherapy drug doxorubicin. In that study, we identified 29 changes in gene expression as the cells acquired resistance to doxorubicin, one major change being the strongly elevated expression of the 1C aldo-keto reductases. We also showed that doxorubicin localization changes from the nucleus to lysosomes as cells acquire doxorubicin resistance (by some unknown mechanism) and that an inhibitor of the 1C aldo-keto reductases augmented doxorubicin cytotoxicity (also by an unknown mechanism). The current manuscript describes a full genome profiling of changes in gene expression accompanying the acquisition of drug resistance. In the current study, 2063 genes were identified as changing expression upon acquisition of doxorubicin resistance under stringent conditions (FDR 0.01, p<0.01). We show that genes or gene families associated with doxorubicin pharmacokinetics (in particular the aldo-keto reductases) are “over-represented” in the dataset and this provides a useful model of how genes that affect doxorubicin pharmacokinetics play an important role in the doxorubicin resistance phenotype. These are “the novel insights into the mechanisms of doxorubicin resistance” to which Dr. Collie-Duguid refers in her review as a strength of our manuscript. We do, however, reveal much more in our
manuscript. For example, we compare the properties of doxorubicin versus doxorubicinol (the product of aldo-keto reductase action on doxorubicin) in terms of their cytotoxicity, DNA-binding activity, and subcellular localization. We show for the first time that, for MCF-7 cells, doxorubicinol has 1 million fold less cytotoxicity to doxorubicin and has significantly reduced DNA-binding activity. In addition, we show that doxorubicinol does not localize to the nucleus in both wildtype and doxorubicin-resistant cells, suggesting that conversion of doxorubicin to doxorubicinol blocks the ability of the drug to reach its target. We also reveal for the first time that two different aldo-keto reductase inhibitors (beta-cholanic acid and flufenamic acid) can increase cellular doxorubicin content in doxorubicin-resistant cells and can restore doxorubicin cytotoxicity and doxorubicin localization to the nucleus in these cells. The above are all significant and novel findings that we feel merit publication in your journal.

b) Four way ANOVA Parameters used in our study: Dr. Elaina Collie-Duguid also requested that we describe the parameters included in the 4-way ANOVA we used to identify significant changes in gene expression associated with the acquisition of doxorubicin resistance. The four parameters were the cell line studied, the dye used to label ribonucleotides, the experimental batch, and the individual arrays used in the microarray study. We have included in the Materials and Methods a clear description of the approach (method of Moments) and parameters used in our 4-way ANOVA.

c) Validation of Microarray Data by RTqPCR: Contrary to Dr. Collie-Duguid’s reasonable interpretation, we did NOT use quantitative RTqPCR to validate our microarray data. Rather, we recognized that the probes on the Agilent whole genome microarray were not isoform-specific for highly-conserved genes such as the aldo-ketoreductases. Hence, we designed isoform-specific probes that would enable us to use RTqPCR to accurately quantity transcript levels of individual AKR isoforms. That’s why AKR1B1 was identified as changed in microarray experiments (along with AKR1B10), since it shares sequence homology with AKR1B10, but only AKR1B10 was validated by RTqPCR. Similarly, AKR1C1 shares sequence homology with the other 1C aldo-ketoreductases. Hence, it was revealed as positively changing gene expression by microarray analysis, while RTqPCR clearly shows that only AKR1C2 and AKR1C3 have altered expression in doxorubicin resistance. We have now made this clear in our manuscript. Thus, it is important to note that our RTqPCR data was not used for array validation. Thus, our RTqPCR findings cannot be interpreted as evidence of a high false discovery rate.

d) Numbers of genes changing expression upon acquisition of doxorubicin resistance: Dr. Collie-Duguid expressed concern that thousands of genes change expression upon acquisition of doxorubicin resistance and wondered whether there was significant “noise” in the dataset, such that many of the changes in gene expression are unrelated to the resistance phenotype. We have conducted multiple full genome microarray studies on a variety of MCF-7 breast tumour cell lines that have acquired resistance to various chemotherapy drugs. In virtually all of our experiments, we identified thousands of changes in gene expression accompanying the acquisition of resistance to chemotherapy agents. We believe these changes in gene expression are “real” since we used stringent parameters in our analysis (p<0.01 AND the false discovery rate was set as 0.01 or 1 in 100). Many of the identified changes in gene expression “make sense”, in particular for genes associated with doxorubicin pharmacokinetics. Nevertheless, we acknowledge that a large number of genes may not be the “drivers” of doxorubicin resistance, but change expression through the altered expression of the drivers. We have made this clear in our revised manuscript.
e) *Overlap/Enrichment with PK/PD genes:* Dr. Collie-Duguid argued that the overlap/enrichment with the PK/PD gene sets does not reach statistical significance when identical genes are compared, but do reach significance when different genes of the same family are considered. She argues that this does not appear to be a valid approach since the potential redundancy of their functions are not explored and no p value is provided in Table 2. We did, in fact, compute p values for this analysis, but the p values are presented in Table 3, not Table 2 (Supplementary Table 2 in the current manuscript). The over-representation is just significant (p=0.05) when exact identifies of genes are considered matches. If we include matches to gene families, the p value is highly significant at <0.001. We used a false discovery rate of 0.01, which is also described in the table. Dr. Collie-Duguid is, nevertheless, correct that while we expect redundancies in genes of similar families, we have not assessed whether this is, in fact, the case.

f) *Interpretation of Microarray findings:* The reviewer also argues that the conclusions regarding the putative role regarding other genes identified in the array analysis are speculative, as only array data is available and that the RTqPCR data demonstrates that the false discovery rate was high. The false discovery rate for our analysis was set at 0.01 (quite low) and as argued above, our RTqPCR experiments did not provide evidence to support a high false discovery rate. Moreover, the over-representation of genes associated with doxorubicin pharmacokinetics suggests the identification of biologically significant genes, in particular since the direction of change in gene expression in each instance would be expected to contribute to doxorubicin resistance. Thus, while admittedly speculative, our microarray study does provide novel insights into likely mechanisms of doxorubicin resistance involving altered expression of genes associated with doxorubicin pharmacokinetics and pharmacodynamics.

g) *Minor essential revisions:*

i) We thank Dr. Collie-Duguid for noting the lack of the * and ** symbols to denote the significant changes in gene expression depicted in Figure 3A. We have corrected this omission.

ii) Tables have now been labelled as supplementary tables. We changed the naming of the tables, since table 1 in the original manuscript occurs in the Materials and Methods section after tables 2, 3, and 4. We have now labelled the tables in the order they appear within the manuscript.

iii) The lysotracker images requested by the reviewer are already published in our *Pharmacogenetics and Genomics* paper. Thus, we feel it is not necessary to include these images as part of a supplementary figure, since these images are already published and subjected to copyright. As for the mitotracker images, these were negative findings (no changes in staining pattern upon acquisition of doxorubicin resistance). Thus, we feel they are not necessary to include in a supplementary figure.

iv) The typographical error in the spelling of the abbreviation for the carbonylreductases (CRs instead of CBRs) has been corrected.

v) Figure 5 communicates a great deal of new information on the degree of uptake of doxorubicin and doxorubicinol in wildtype and doxorubicin-resistant cells, on the effect of 5β-cholanic acid on this uptake of the two agents in both cell lines, the localization of these agents in wildtype and doxorubicin-resistant cells, and the effect of 5β-cholanic acid on this localization. These are important findings that document how changes in the expression or activity of one doxorubicin pharmacokinetic gene alone can exert dramatic changes in the drug’s properties. Thus, these findings warrant significant coverage in the manuscript.

vi) We agree with Dr. Collie-Duguid that we cannot conclude that there is a 2-fold increase in doxorubicin content when MCF-7_CCl12 cells are treated with both cyclosporine A and 5β-cholanic acid. We state in the
manuscript that this observation is not statistically significant, thereby questioning the validity of our findings.

vi) We also agree that we cannot conclude that the effects cyclosporine A on doxorubicin content are due to the overexpression of Abcc1, since cyclosporine A is a pan-ABC inhibitor. We have thus modified the wording to state: “These differences relative to untreated cells were found to be highly significant, and are likely due to the increased expression of AKRs [17] and ABC drug transporters known to be over-expressed in MCF-7_{DOX2-12} cells, such as Abcc1 [27].”

Review by Martin Clynes:

We thank Dr. Clynes for his positive comments that our manuscript would represent a useful addition to the literature and that he recommends acceptance of our manuscript for publication. We have corrected the one minor typo in the spelling of one of our cell lines in the Results section of our manuscript.

We again want to thank Drs. Collie-Duguid and Clynes for their learned review of our manuscript. We feel that our manuscript is significantly improved as a result of this review. We trust that the changes we have made in our revised manuscript have effectively addressed the comments and concerns of these reviewers and that our manuscript is now acceptable for publication in *BMC Cancer*. I look forward to your reply in the near future.

Yours sincerely,

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