Author's response to reviews

Title: Metformin-mediated growth inhibition involves suppression of the IGF-I receptor signalling pathway in human pancreatic cancer cells

Authors:

Emelie Karnevi (emelie.karnevi@med.lu.se)
Katarzyna Said (katarzyna.said@med.lu.se)
Roland Andersson (roland.andersson@med.lu.se)
Ann H Rosendahl (ann.rosendahl@med.lu.se)

Version: 2 Date: 22 January 2013

Author's response to reviews: see over
Dear Editor,

MS: 1583659604822402 “Metformin-mediated growth inhibition involves suppression of the IGF-1 receptor signalling pathway in human pancreatic cancer cells” by Emelie Karnevi et al.

Enclosed please find the revised manuscript, changed according to the comments and questions made by the reviewers. The changes made in the text are underlined. A revised version of the manuscript has been accepted by all co-authors for resubmission to BMC Cancer.

We hope you will find the revised paper changed according to suggestions and hopefully now acceptable for publication. We are grateful for the comments and suggestions from the reviewers. Our detailed answers and where they are to be found in the manuscript are listed below.

Sincerely yours,

Roland Andersson

Roland Andersson, MD, PhD
Professor of Surgery
Department of Surgery, Clinical Sciences Lund
Skåne University Hospital
SE-221 85 Lund, Sweden

/mk
Reviewer 1:
(Enrique Rozengurt)

1) The concentrations of metformin used in this report are very high (10-20 mM) and the significance of effects obtained at such high concentrations is questionable.

Re: The doses of metformin used in the present study lie within the dose range applied in the majority of published experimental in vitro studies using metformin. The translation of in vitro doses to an in vivo study or to human subjects most often require a 10-100-fold dose reduction, due to the enhanced local sensitivity in vivo. We agree with the reviewer that it is a valid point to monitor the effects of metformin also at lower doses in additional studies. An in vitro study by Sinnett-Smith and colleagues was recently published, demonstrating an effect by metformin at the low 0.05-1 mM range in MIAPaCa-2 and PANC-1 pancreatic cancer cells. This study further supports our present data, demonstrating a direct effect of metformin on pancreatic cancer cells and that the sensitivity to metformin is significantly improved at physiologically normal concentrations of glucose. This reference [33] has now been included in the revised manuscript and is discussed further on page 13, line 7.

2) In Fig. 1, was the expression of LKB1 in AsPC-1 confirmed to be silenced? There are clonal variations in this cell line and some AsPC-1 show substantial expression of LKB1.

Re: The methylation status and expression of LKB1 in AsPC-1 was not evaluated in the present study. In the revised manuscript we have rephrased the description in the discussion, page 12, line 21. It now reads “AsPC-1 cells have previously been reported to carry an epigenetic inactivation of LKB1 (ref 27)”.

3) The function of the phosphorylation of AMPK at Ser485 is not clear in the literature and this paper does not contribute to clarify this issue. The authors cite a previous paper in which the phosphorylation of Ser485 hinders that of Thr182. However, a different study reported that mutants of Ser485 that either prevent phosphorylation (Ala substitution) or mimic phosphorylation (Asp substitution) did not affect the overall AMPK activity, so it was concluded that phosphorylation of this residue did not participate directly in the regulation of AMPK activity [J. Biol. Chem., 278 (2003), pp. 28434–28442]. The notion that phosphorylation of Ser485 impairs that at Thr182 has been challenged recently by another group [FEBS Letters, Oct 29 2012]. The manuscript does not make a significant contribution to the role of these phosphorylation because no mutant results are presented and thus, the paper describes changes of phosphorylation rather than provides any insight into the mechanisms involved.

Re: We agree with the reviewer that the role of Ser485 in the complex AMPK signalling network is at present not completely clear in the literature. The existing conflicting data on correlations between AMPK^Thr172 and AMPK^Ser485 phosphorylation, suggest that there may be cell- and context-related variations. These variations further appear to relate to both differential physiological activation (e.g. PKA vs. PKB/Akt-induced) as well as to differences between physiological and pharmacological activation of the Thr172 and SerS485 residues. The paper by Garcia-Haro et al (FEBS Letters, 2012) proposes that endogenous PKA-induced activation of AMPK^Ser485 does not affect the phosphorylation status of Thr172. However, the authors further discuss that PKB/Akt-induced activation at Ser485 may prevent the phosphorylation status of
Thr172, in line with our present study. This reference [34] is now included in the revised manuscript and is discussed on page 13, line 14.

4) More important, the results in Fig. 3 show the effect of metformin on the phosphorylation of AMPK on Ser485 is completely different (in fact the opposite) in the cell lines studied (compare AMPKser485 in panel A, with AMPKSer485 panel D, 5 mM glucose).

Re: This is a correct observation by the reviewer and relate to the above mentioned point. However, the main aim of this study was to examine direct anti-tumor effects by metformin in the context of high and low glucose. Most importantly, although the discrepant AMPKSer485 profile at low glucose, exposure to metformin induced a similar activation pattern of AMPKThr172, the main activation site mediating the biological effects of metformin, and anti-tumor effect in both cell lines. We agree that further studies are warranted to clarify the biological role of AMPKSer485 at normal glucose levels. This is discussed on page 13, line 14.

5) In Fig. 6, metformin did no inhibit IGF-induced pAkt in MiaPaCa-2 at either 5 or 25 mM glucose. Given that the title of this paper revolts around suppression of IGFR signalling by metformin, the results with MiaPaCa-2 in Fig.6 do not support the conclusion of the study.

Re: The quality of the Western blot for pAkt in MIAPaCa-2 has been improved to more clearly demonstrate the reduction in IGF-I-induced pAkt following metformin treatment at 5 mM, but not at 25 mM glucose. This is further verified in the densitometry quantifications of three individual experiments showing a significant reduction of the IGF-I-induced pAkt by metformin at 5 mM glucose in both BxPC (Fig 6C; P<0.01) and MiaPaCa-2 (Fig 6F; P<0.001) pancreatic cancer cells.

6) The perception that the paper is descriptive rather than mechanistic is reinforced by the fact that no studies are presented attempting to clarify why the effects of metformin appear to be increased in cells incubated in medium with 5 mM rather 25 mM glucose.

Re: Several previous studies have demonstrated the importance of AMPK activation at Thr172 for the pharmacological effect of metformin. In line with this, the present study demonstrates a significant activation of AMPKT172 associated with a significant anti-proliferative effect of metformin at physiological normal glucose levels. In sharp contrast, the metformin-induced AMPKThr172 activation was significantly impaired at high glucose resulting in significantly less growth inhibition. In addition, the present study demonstrates a significant inhibition of the growth- and survival promoting IGF-IR pathway by metformin at 5 mM glucose, with reduced IGF-IR activation as well as downstream effectors IRS-1 and pAkt. In contrast, levels of pIGF-IR, IRS-1 and pAkt remained activated at high glucose. Taken together, these findings suggest that both the impaired AMPKThr172 activation as well as sustained IGF-IR/Akt activation may contribute to the reduced sensitivity to metformin at high glucose.

7) In general, none of the experiments involving western blot analysis show standard errors. Where these experiments performed only once? The quality and results obtained
with the western blots needs improvement. Examples: AMPK in Fig. 3 D, Akt in Fig 4A (5 mM), Akt in Fig 6 A (25 mM).

Re: We thank the reviewer for these constructive comments. In the revised manuscript, the densitometry quantifications representing means ± SE of three individual repeats of Western blot analyses have been included for figures 2-6. We agree with the reviewer and believe these quantifications strengthen our findings. In addition, the qualities of the representative western blots shown have been improved as requested by the reviewer.

Reviewer 2:
(Elisabeth Barton)

Major Compulsory Revisions (Specific points)

1. The goals of the study need to be more clearly stated in the introduction. They are too descriptive.

Re: As suggested by the reviewer, we have rephrased the last paragraph of the introduction (page 5, line 18) to clarify the specific aims of the study.

2. While many of the figures show compelling data in the form of immunoblots, there is no quantification or statistical analysis provided. Thus, the results in Figures 2-6 are anecdotal, and the bar graphs are only descriptive of 1 replicate. All of these results must be shown in terms of the multiple replicates performed, to ensure that the blots shown reflect the main conclusions.

Re: We thank the reviewer for these constructive comments. In the revised manuscript, the densitometry quantifications representing means ± SE of three individual repeats of Western blot analyses have been included for figures 2-6. We agree with the reviewer and believe these quantifications strengthen our findings.

3. The dose of metformin seems higher than those used in other publications (1-5 mM appears in several culture studies). Is there a reason for such a high dose? Further, it is not clear what does was utilized for the experiments in figure 5 and 6 – 10 or 20 mM? Are there off-target effects associated with such high concentrations? For instance in Panels of Figure 1, B and C exhibit no dose dependence, whereas A shows some modest does differences between 10 and 20 mM. Where are these effects saturated?

Re: The doses of metformin used in the present study (10-20 mM) lie within the dose range applied in the majority of published experimental in vitro studies using metformin (5-60 mM in most previous studies). The translation of in vitro doses to an in vivo setting or to human subjects most often require a 10-100-fold dose reduction, due to the enhanced local sensitivity in vivo. To avoid potential saturation effects, the lower 10 mM dose was used in all experiments and is presented in the figures. The metformin dose (10 mM) used in figures 5 and 6 is now more clearly indicated on the figure panel and legends. We agree with the reviewer that it is a valid point to in additional studies monitor the effects of metformin also at lower doses. An in vitro study by Sinnett-Smith and colleagues was recently published demonstrating an effect by metformin at the low 0.05-1 mM range in MIAPaCa-2 and PANC-1 pancreatic cancer
cells. This study further supports our present data, demonstrating a direct effect of metformin on pancreatic cancer cells and that the sensitivity to metformin is significantly improved at physiologically normal concentrations of glucose. This reference [33] has now been included in the revised manuscript and is discussed further on page 13, line 7.

4. There are no descriptions of the statistical analyses other than proliferation. This is related to the 2nd point, where there are only blots with no quantification shown.

Re: Statistical analyses have now been included for the quantified Western blots and in the Results and Methods sections.

5. For Figure 2, there was 1% serum added in this experiment, but the rationale is not clear.

Re: In order to examine the effect by metformin with a basal activation of intracellular growth and survival pathways, 1% serum was added in these experiments.

Minor Essential Revisions (Minor comments)

1. The data presentation in Figures would be improved if the cell lines used were notated on the panels.

Re: The cell lines are now indicated on the panels of the figures.

2. Please define SFM (I assume it is serum free media) in the methods.

Re: SFM has now been defined in full in the methods.

3. Page 4, line 6. Suggest replacing “up to” with “almost”.

Re: This has now been done.


Re: This has now been done.

5. A recent paper on this subject was published using the same cell lines, and glucose modulation (Sinnett-Smith et al, 2012, BBRC). While this might have not been available prior to the authors submission, it will be important to address the findings in this study with respect to this publication.

Re: As indicated by the reviewer, the recent paper by Sinnett-Smith and colleagues was submitted to BBRC after the submission of our present study and was thus not publicly available. The findings of this paper are in concordance with our data demonstrating direct anti-tumour effects of metformin and support our findings of enhanced sensitivity
at physiological normal glucose levels. This reference has now been included [33] and is further discussed on page 13, line 7.

**Quality of written English:** Needs some language corrections before being published

*Re:* The manuscript has been proofread by a native English speaker and changes made accordingly.