Title: Glycogen Synthase Kinase-3 Inhibition Disrupts Nuclear Factor-kappa B activity in Pancreatic Cancer, but Fails to Sensitize to Chemotherapy

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Author's response to reviews: see over
Dear Dr Marshall

Re: MS ID: 4686468482176962 “Glycogen Synthase Kinase-3 Inhibition Disrupts Nuclear Factor-kappaB Activity in Pancreatic Cancer, but Fails to Sensitize to Chemotherapy”, by Mamaghani et al. We appreciate the referees’ detailed comments about this manuscript. Many of these relate to the molecular mechanisms of GSK3 action. Our collaborator at the University of Toronto, Jim Woodgett, is one of the world’s leading experts on GSK, and we have now included one of Jim’s senior postdocs, Satish Patel, on the authorship. Satish has been a major resource throughout this project and has agreed to be a co-author. We have done additional experiments as requested and made extensive changes itemized as follows:

Reviewer: Dr. Heiner Schäfer

Overall, an interesting paper but the experimental data provided by the study are not sufficient as to fully support the conclusions. Thus some improvement and additional experiments are needed. Moreover, the inconsistent use of some cell lines (Panc1 and BxPc3 in figure 1; Panc1 and MiaPaca-2 in figure 2, 5 & 6; BxPc3 and MiaPaca-2 in figure 4a; BxPc3 and Panc1 in figure 4c) is a bit annoying.

This project is based on a total of 6 pancreatic cancer cell lines that broadly show similar responses as described in the text of the manuscript. We used Panc-1 and BxPC-3 extensively during the initial characterization of effects of GSK3 inhibition, but found that BxPC-3 was difficult to transfect, so used MIA PaCa-2 and PANC-1 for the experiments using RNA silencing and reporter constructs.

Major compulsory revisions:

1. The role of GSK3 in NFkappaB regulation is not fully clear. What about the negative effect of GSK3 dependent Ser468-phosphorylation of p65/RelA on basal NFkappaB activity in the pancreatic cancer cell lines? Since the basal rather than the inducible NFkappaB activity may determine chemoresistance this issue has to be addressed.

We agree with the reviewer that the basal level of NF-κB activity might be the determining factor of chemoresistance, and that is why all of our studies (including immunoblotting, SRB assay and luciferase reporter assay) revolve around the reduction of basal rather than inducible NF-κB activity, and ask if the cells are sensitized when these levels are reduced following GSK-3 inhibition. There are a number of studies indicating inhibitory (through Ser468 phosphorylation of p65 subunit), as well as stimulatory effects (through transcriptional activation) of GSK-3 on NFκB. We review this in our Introduction, and have also made some additional changes to emphasize this point. Our data clearly point to a positive role for GSK-3 supporting NFκB in pancreatic cancer cells. This effect has been recognized for almost 10 years in different models. However, the underlying mechanisms remain uncertain. The suppressing effects of GSK-3 inhibition may be in part explained by the fact that GSK-3 inhibition stabilizes β-catenin and β-catenin in turn can inhibit NF-κB.

Furthermore, previous studies that we cite indicate that pancreatic cancer cell lines, as well as patient biopsies, contain a high level of basal NF-κB activity. Although we cannot exclude the possibility that GSK-3 might also be having some inhibitory effect through Ser468 phosphorylation, the overall effect of GSK-3 inhibition is to reduce NF-κB
activity in all cell lines tested. We believe that the regulatory effect of GSK-3 on NF-κB is likely context- and cell type-dependent, and the exact mechanism of regulation is yet to be identified.

2. Since the observed biphasic effects with gemcitabine reflect cell cycle dependence, the authors need to include cell cycle analysis. What is the effect of GSK3 inhibition on the cell cycle? If it is blocked, the cytotoxic effect of gemcitabine might be reduced by GSK3 inhibition thus masking an otherwise sensitizing effect due to the downregulation of anti-apoptotic genes.

We agree with the comment that since gemcitabine cytotoxicity is cell cycle-dependent, GSK-3 inhibition might be antagonistic if this resulted in cell cycle arrest. We discuss this point at the bottom of Page 20 of the revised manuscript. Several GSK-3 inhibitors are also cyclin-dependent kinase inhibitors, although AR-18 is reported not to have this effect. In our studies, we found that the cell cycle effects of AR-18 were much less evident than those with the unrelated GSK-3 inhibitor SB216763, which produced G2 arrest. However, we did see a minor reduction in S-phase cells following exposure to AR-18.

To address the reviewer’s concern, we tested the interaction between AR-18 and gemcitabine by sequential treatment of cells with both the drugs. Briefly, PANC-1 and BxPC-3 cells were treated with increasing doses of gemcitabine (0.05, 0.2, 0.5, and 1µM) for 48 h and subsequently were incubated for 24 h with GSK-3 inhibitor AR-18 in 200:1 ratio as described previously in materials and methods section of the paper. Consistent with our previous results where we performed a concurrent treatment of both agents, there was sub- additive effect observed across the higher doses of both drugs. We also studied the effect when AR-18 was added 24 h prior to incubation with gemcitabine, and did not see sensitization.

3. The appr. 2-fold induction of NFκappaB driven luciferase in the cell lines by TNFalpha is rather low. Thus, there seems to be some limitation with the NFκappaB read-out as to accurately assess the impact of GSK3 inhibition. What is the effect of the scrambled siRNA on TNFalpha induced luciferase (Figure 3b)?

The relatively low levels of NFκB induction by TNFα are explained by the high basal activity seen in the pancreatic cancer cells. As seen for example in Figure 3, the dynamic range of this assay is approximately 5-fold when one examines the low levels achieved during GSK-3 inhibition, which is similar to the range reported by other groups using this technique.

The effect of scrambled siRNA on TNFα induced luciferase was shown on the original figure 3 as the second bar in the bar graph, but the labelling of this figure was unclear, and we have modified this in the revised paper.

Minor Essential Revisions:

1. The use of proliferation assays seems to be inconsistent. In Figure 1, the assay method was not specified (neither in the text on page 12 nor in the figure legend) and the data refer to "relative cell proliferation", whereas in figure 4a and 6b the results refer to "relative optical density". This should be clarified.

We agree that the terminology used to describe the results of the SRB was inconsistent in the original version of the manuscript, and have changed this to "relative cell proliferation" throughout, as suggested.
2) For their sensitization experiments, the authors need to include GSK3 knock down when analysing GSK3 inhibition in combination with gemcitabine.
As shown in Figure 5b, we tested the interaction between GSK-3 knockdown and gemcitabine using the NFκB luciferase reporter. We agree with the suggestion to test if GSK-3 knockdown sensitizes to gemcitabine, but found it technically difficult to test this due to the transient nature of GSK-3 knockdown, and inefficient transfection in 96 well plates.

3) Figure 1 a, left panel: Typo in the x-axis labelling.
We made the changes in the axis.

Discretionary Revisions:
1) Addressing the clear downregulation of anti-apoptotic genes by GSK3 inhibition, more specific apoptosis assays should be included in the study.
We used several methods to test if inhibition of GSK-3 triggers apoptosis in pancreatic cancer cells (PARP cleavage, loss of mitochondrial membrane potential, rescue using the general caspase inhibitor zVAD), and surprisingly did not find convincing evidence that apoptosis was taking place. We comment on this in the Discussion, bottom of Page 21. Since these are negative findings, we did not show these results but we can do so if requested. In our previous publications we have been able to detect apoptosis when these cells were exposed to other agents, so we do not believe this finding is explained by inability of these cells to undergo apoptosis, or technical problems with the laboratory techniques. The mode of cytotoxicity remains uncertain, but appears not to involve classical apoptosis.

2) When using AR, basal level of XIAP but not of Bcl-XI were downregulated in Panc1 cells, whereas GSK3 knock down in the same cell line only affected Bcl-XI but not XIAP expression. How these distinct effects could be explained?
The reviewer is referring to the blots shown in Fig 2A+B, versus 3A. With both siRNA and AR-18 we see increased β-catenin, confirming GSK-3 inhibition. This is associated with reduced XIAP and Bcl-XI in both cases, although we agree that with AR-18 treatment, the effect is greater with XIAP, whereas with siRNA has more effect on Bcl-XI. Although it is not clear why there is this differential effect, we suspect that it might be explained by differences in the kinetics of GSK-3 inhibition with the two agents.

Moreover, how GSK3 inhibition affects the inducible (i.e. by TNFalpha) Bcl-XI and XIAP expression? CHIP assays would be appropriate to analyse the effect of GSK3 inhibition on NFκappaB binding to the XIAP and Bcl-XI promoters.
This is an interesting suggestion, but in this paper we used TNFα as a positive control for the NFκB reporter, and our intent was not to study the effects of the activation of target genes like XIAP.

3) How GSK3 inhibition affects the sensitivity of PANC1 cells against the cytotoxic effect of TNFalpha or other apoptotic stimuli? What about other anti-cancer drugs such as 5-FU?
We agree that it might be of interest to test if GSK-3 inhibition sensitizes pancreatic cancer cells to other cytotoxic agents like 5-FU. However, these agents have very limited clinical activity and we decided not to pursue this further, given the results obtained with
gemcitabine. In contrast, we agree that the disruption of NFκB by GSK-3 inhibitors might sensitize to other apoptotic stimuli, and are actively studying this. However, this is a large undertaking that we do not believe appropriate for the present paper.

Reviewer: Dr. Michele Yip-Schneider

Major compulsory revisions

1. Figure 4A and page 14: Although AR-18 shows a dose response curve, gemcitabine displays a plateau. The authors should try lower doses of gemcitabine (<0.05 μM) at the longer time point of 72 hours to obtain a dose response curve. Published work shows that very low concentrations of gemcitabine are effective in pancreatic cancer cells. It is also possible that the 200:1 drug ratio is not optimal for detecting efficacy of the combination. How was this ratio determined? Perhaps other dose combinations should be evaluated to exclude a detection problem.

We agreed with these comments and performed additional experiments as suggested. Gemcitabine is a cell cycle phase-dependent agent, where the toxic effect is determined by the duration of exposure as well as the drug concentration. This is readily seen in our data, and we have emphasized this point in the revised manuscript (top of page 15). As suggested by the reviewer, we extended the dose response and tested gemcitabine concentrations from 0.01-10 μM for 24, 48 and 72 hrs. For PANC-1 cells we do see a synergistic interaction between gemcitabine 0.01 μM and AR-18 at 48hr, as was shown in our original Fig 4B, and we have modified the manuscript at several places to point out that under some circumstances synergy can be obtained. Interestingly, at the 72hr time point suggested by this reviewer the effect was only additive. We have performed extensive testing involving different ratios of gemcitabine and AR-18, as well as the fixed 200:1 ratio, similar to the results shown in our original Figure 4. As well as the SRB proliferation assay, we also tested for effects of drug treatment on clonogenic survival. With the exception of the PANC-1 results, we have not been able to show drug synergy. In many cases there was an additive effect, but we also found many instances where the effect was sub-additive or even antagonistic. Thus we hold to our position that despite the reasonable hypothesis that NFκB inactivation would lead to gemcitabine sensitization in pancreatic cancer cells, this effect is fairly minor and cell line dependent.

2. Figure 4B and page 14: In PANC-1 cells, the combination appears to be more effective than the single agents. Gemcitabine at the 0.01 and 0.1uM doses shows a relative optical density of 100, while the combination shows a ~20% decrease in optical density compared to corresponding AR-18 doses alone. This suggests greater than additive or possibly synergistic effects of the combination in this one cell line.

As for the first comment by this reviewer, we agree that our data do show a positive interaction in PANC-1 cells, and this finding was not sufficiently commented on in the original manuscript. We have therefore modified the revised text (middle of page 15 and bottom of page 19).

3. Figure 5 and page 15: The authors contrast their results with that of a previous study in which gemcitabine was shown to induce NF-κB using a gel shift assay. The authors should confirm their NF-κB reporter assay results by gel shift as well since they mention
that their results depend upon experimental conditions. There may be some technical issues with the reporter assay, i.e. possible low transfection efficiency of cells etc.

The previous paper by Arlt et al referred to shows a relatively small increase in NFκB activity using a luciferase assay similar to the one used in our laboratory. However, it should be noted that these authors used 20µM gemcitabine to achieve this result, whereas lower drug doses were much less effective. Therefore our own results, which do show a modest but statistically significant NFκB activation in PANC-1 cells treated with 10µM gemcitabine, appear to be consistent with the Arlt et al paper. We have modified the text to make this comment (middle of Page 16). However, this appears to be cell line dependent as we did not see NFκB activation in MiaPaCa-2, PK1, or PK8 cells under similar experimental conditions.

To test the transfection efficiency, we co-transfect the cells with Beta galactosidase vector bearing CMV promoter. The results are measured by dual luciferase assay, indicating the efficiency of the transfection. Also, the luciferase results are normalized against the measured beta galactosidase activity to exclude a transfection efficiency problem. Therefore we do not believe there is a technical problem with the luciferase reporter system.

4. The hypothesis of this paper is that if GSK-3 inhibition suppresses the NF-kB pathway which plays an active role in resistance, then GSK-3 inhibition should sensitize pancreatic cancer cells to gemcitabine which has been shown to activate NF-kB. The lack of observed synergy (figure 4) may be due to the absence of NF-kB induction by gemcitabine in these lines (figure 5 for PaCa-2 and Panc-1, reporter assay). Does gemcitabine induce NF-kB in the other cell lines, BxPC-3, PK-1, -8 or HPAC?

The hypothesis is stated correctly. However, we are not claiming that NFκB is being activated by gemcitabine treatment, as implied by the reviewer. As shown Figures 2 and 3, the pancreatic cancer cell lines show constitutive NFκB activity that is inhibited by GSK-3 inhibition or silencing. In PANC-1 there is a modest increase in NFκB following gemcitabine, but not in the other cell lines tested. This is discussed in our response to comment #3.

As we mention in our Introduction (Page 4, second paragraph), constitutive activation of NFκB has been well documented in pancreatic cancer. The mechanisms for this are not clear, but are likely multifactorial. For example, pancreatic cancers show activation of signalling pathways that can activate IKK, high levels of oxidative stress, and release of death receptor ligands; all of which can activate NFκB.

5. Relatively high doses of the agents were chosen for some of the assays – AR-18 (50uM), curcumin (50uM) and gemcitabine (10uM). How were these doses chosen?

Initial selection of the doses used in the luciferase assay was based on the previous literature. We also tested a wide range of lower concentrations, particularly for growth inhibition and clonogenic survival. Final selection of doses was based on these experiments, as shown in Figures 4 and 6.

6. The authors could not replicate the work of Kunnumakkara showing that curcumin sensitizes pancreatic cancer cells to gemcitabine. Again, this may be a technical issue in choosing set ratios of drugs to test. Kunnumakkara employed 10uM curcumin and 50nM gemcitabine. This same dose combination should be tried before conclusions can be made.
We repeated this experiment using the exact doses and treatment schedule (i.e. 4h pretreatment with curcumin) described in the Kunnumakkara paper, as suggested by the reviewer, as well as a range of additional curcumin doses. However, at best we found a modest additive effect at the lower doses of curcumin. In our hands, the combination of 10µM curcumin and 50nM gemcitabine was actually antagonistic. Similar results were obtained with PANC-1 and MIA PaCa-2 cells, consistent with our earlier results described in the original manuscript. The reason for this discrepancy is not clear, and might reflect differences in experimental conditions that were not accounted for.

Reviewer: Dr. Richard Bold

Major Revisions:

1. Much of the work centers around use of AR-18 to inhibit GSK. The pharmacology of this compound as described by R Bhat et al (JBC, 2003) suggests an inhibition of GSK3 in the mid nanomolar range, and cellular effects (cell death) with an IC50 of 0.5 micromolar. The current study notes an IC50 of nearly 100-fold higher (35 micromolar) with little observed cellular effects at doses up to 10 micromolar (20-fold higher than the IC50 noted by Bhat et al.). Since this is a relatively new compound, in order to specifically link GSK3 inhibition to the observed results, it would be beneficial to show a correlation of GSK3 inhibition pharmacodynamics with the cellular events.

The mid nanomolar inhibition of GSK-3 reported by Bhat et al was obtained using an in vitro kinase assay, whereas drug effects in cell-based assays required higher doses. In their paper they used several different biological endpoints to measure drug effects, mainly based on neuronal cells, and in some of these the doses were similar to the ones being used in the present paper. We have used cytoplasmic accumulation of β-catenin as our pharmacodynamic readout, which we feel is appropriate since this is not directly related to the effects on NFκB.

2. The dose-dependancy of the cellular response of these cells (Figure 1B) is very similar. Is the activity of GSK3 (by kinase assay) and/or NF-kB activity of these cell lines similar? This would tie the inhibition of the target with the mediator together nicely, though other studies may have suggested that these cell lines have differing NF-kappaB activity.

We considered the interesting suggestion to compare the activities of GSK-3 kinase and NFκB. However, we were not able to design an experiment to test this, since the available kinase assay requires an enzyme isolation procedure that we would expect to reverse target inhibition, given that AR-18 is not an irreversible GSK-3 inhibitor. This comment is partly addressed in the use of β-catenin accumulation as a readout for GSK-3 inhibition.

3. The data of Figure 2C in which AR-18 blocked TNF-mediated NF-kappaB is intriguing, suggesting that TNF activates NF-kappaB through a GSK3 mechanism, or that the inhibition is so potent by AR-18 that activation cannot occur. The authors should specifically address these issues.

The effect of GSK-3 inhibition on TNFα-mediated NFκB in the pancreatic cancer cells is consistent with the previous work by Hoeflich et al describing effects of GSK-3 knockdown in normal hepatocytes (reference 26), and not a novel finding. The molecular mechanisms for this effect remain uncertain and somewhat controversial. However, likely GSK-3 is not responsible for the initial activation and nuclear translocation of
NFκB, but instead serves to maintain transcriptional activity. We have modified the Discussion (top of Page 18) to emphasize this point, and have included references to the earlier literature.

4. In Figure 3B, there is only modest effect on XIAP with combined knockdown, despite dramatic XIAP inhibition with AR-18 (Figure 2). These data should be reconciled. This point was also noted by Reviewer #1. We think it likely that the effects on XIAP are related to differences in the extent and duration of GSK-3 disruption using siRNA versus a kinase inhibitor. However, it should be noted that decreases occur in XIAP and also in the alternative NFκB target Bcl-XL, supporting the idea that NFκB is being inhibited, as was also shown using the luciferase reporter assay.

5. The observation that inhibition of NF-kB with curcurmin does not sensitize these cells to the effect of gemcitabine is contrary to several papers using both pharmacologic and molecular inhibitors of NF-kappaB. The authors may wish to examine other methods of cell death or cellular response, since their findings are contrary to what has been previously extensively reported. This point was also raised by Reviewer #2. Using the SRB assay we confirmed that curcumin as a single agent was effective against pancreatic cancer cells at dose levels similar to those used by others, as seen in Figure 6. However, we were unable to replicate the sensitization of pancreatic cancer cells to gemcitabine using a range of curcumin doses, including dose schedules that were previously reported to be effective. It should be noted that curcumin is not a specific NFκB inhibitor, and that some of the positive effects noted by others might be due to other biological effects that were not strongly expressed in our experimental models.

Minor Essential Revisions:

1. The font size for the text within the panels of Figure 4 is too small. Necessary changes have been implemented according to the reviewer’s suggestion.

2. It is unclear what the studies using TNf add to the paper, since the supposition is that the GSK is active through epigenetic events, causing the constitutive activation of NF-kappaB.

The main theme of our study has been the basal endogenous levels of NF-κB in pancreatic cancer cells. TNF-α was used for the following reasons:
A) To act as a positive control for induction of NF-κB activity
B) To test the integrity of our luciferase reporter assay
C) To test if our results are consistent with the previous reports placing GSK-3 in a position that could mediate NF-κB activity downstream of TNF-α.
D) Irrespective of the level of activation, we could show that GSK-3 inhibitors can inhibit both induced as well as basal levels of NF-κB activity.

We believe that these revisions have substantially improved and strengthen the paper, and look forward to your response.