p-21 activated kinase as a molecular target for chemoprevention in diabetes

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ABSTRACT

Hypothesis: Anti-diabetic drugs modulate p-21 activated kinase (PAK) signaling

Introduction: Type 2 diabetes mellitus (T2DM) is a chronic inflammatory disease associated with increased cancer risk. PAK signaling is implicated in cellular homeostasis when regulated, and cancer when unrestrained. Recent reports provided a role for PAK signaling in glucose homeostasis, but the role of PAKs in the pathogenesis of T2DM is unknown. Here we explored whether PAK signaling should be targeted via chemoprevention to reduce diabetes-associated cancer risk.

Methods: PAK interacting partners in T2DM were identified using online STRING database. Systematic literature review provided the effect of anti-diabetic drugs on PAK signaling. Review of clinical studies revealed the overall clinical response rate and percentage of adverse events in pioglitazone (n=53) and metformin (n=91) treated patients with PAK-dependent diseases.

Results: A total of 30 PAK interacting partners were identified (10: reduced beta-cell mass; 10: beta-cell dysfunction; 10: obesity-insulin resistance) which were highly associated with Wnt, and G-protein signaling. Anti-diabetic drug metformin activated signaling pathways upstream; whereas pioglitazone inhibited pathways downstream of PAK. Overall clinical response upon pioglitazone treatment was 53%. 79% of pioglitazone and 75% of metformin treated patients had adverse events. Pioglitazone reduced molecular-PAK biomarkers of proliferation (Ki67 and CyclinD1), and metformin had the opposite effect.

Conclusions: PAK signaling in T2DM involves Wnt and G-protein signaling which is altered by anti-diabetic drugs metformin and pioglitazone. Apart from
the therapeutic limitations of adverse events, pioglitazone is promising in chemoprevention, however long-term multi-centered studies, which initiate pioglitazone treatment early will be required to fully assess the full potential of these drugs.

Keywords: p-21 activated kinase; pioglitazone, metformin, type 2 diabetes mellitus, cancer, chemoprevention, and inflammation.
INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a global epidemic and its prevalence is continually increasing, especially in the western world. The etiology of T2DM is intimately linked to obesity, genetics, and sedentary lifestyle. Disease manifestations including blindness and neuropathy decrease quality of life in diabetic patients, and long-term complications such as cardiovascular disease and renal failure, ultimately result in death (McCarthy, 2010).

Although the pathogenesis of T2DM is multifactorial and complex, its current understanding encompasses hepatic insulin resistance, dysfunctional insulin signaling, abnormal glucose metabolism, and persistent hyperglycemia (McCarthy, 2010). In addition to disease specific complications, several consequences of hyperglycemia have been described such as an aberrant immune response, chronic inflammation, and tumorigenesis (Chang & Yang, 2016). Persistent diabetes increases the lifelong risk of developing pancreatic, liver, and colorectal cancer and also fuels the tumor microenvironment in cancer patients (Chang & Yang, 2016).

Anti-diabetic drugs such as the biguanides, sulfonylureas, and glitazones have provided the current basis of understanding in the clinical management of T2DM however little is known regarding whether these drugs are also effective in reducing the associated cancer risk. Effective chemoprevention in T2DM will target processes involved in both glucose metabolism and carcinogenesis.

Molecular targets with emerging roles in both cancer and diabetes include a family of six different kinases, the p-21 activated kinases (PAKs) (Dammann et al., 2014). Here we investigated PAK overexpression in T2DM, the effect of anti-
diabetic drugs on PAK signaling, and further explored PAK as a molecular target in cancer chemoprevention in T2DM.

**METHODS**

Molecular analysis of PAK signaling pathways and their involvement in response to anti-diabetic drugs

A systematic literature review and molecular analysis of pre-clinical studies using online library Pubmed (https://www.ncbi.nlm.nih.gov/pubmed) was performed to establish upstream and downstream PAK targets. Key words included (PAK signaling or p21 activated kinases in addition to the following targets: AMPK, RAS, mTOR, PI3K/AKT, RAC1, CDC42, MAPK, p38, JNK, NF-kB, PPAR-gamma, ROS, VEGF, Wnt/Beta-catenin). Similar studies were screened for PAK signaling pathways involving known targets (above) of glucose homeostasis, inflammation, proliferation, survival, and angiogenesis. Both upstream and downstream PAK targets were identified in each of these pathways, and the effect of PAK signaling targets on inflammation, proliferation, survival, and angiogenesis was evaluated. Studies in the literature involving anti-diabetics drugs (glitazones, metformin, glyburide) were further analyzed for their effect on PAK signaling pathways (inflammation, proliferation, survival, angiogenesis) on targets upstream and downstream of PAKs.

Identification of PAK interacting partners

PAK interacting partners were identified using string database (www.string-db.org) as seen in (Szklarczyk et al., 2015)(Campregher et al., 2012). Confidence was set to 0.40 and active prediction methods, neighborhood, gene fusion, co-
occurrence, co-expression, experiments, database, and text mining analysis were performed. Interacting partners were identified in three different conditions which may predispose to T2DM using targets of reduced beta-cell mass (CDKAL1, CDKN2A, CDKN2B), beta cell dysfunction (MTNR1B, TCF7L2, KCNJ11), and obesity/insulin resistance (FTO, IRS1, PPARG) as reported (McCarthy, 2010). Novel PAK interacting partners were further investigated and their pathophysiological role in T2DM was investigated and references for further exploration of targets were provided. All PAK partners were analyzed according to the molecular pathway involved in T2DM.

Clinical study inclusion/exclusion criteria and systematic review

Human clinical studies were identified on ClinicalTrial.gov. Inclusion criteria consisted of observational or interventional studies using metformin or pioglitazone in patients with diseases previously identified in the literature to have increased PAK expression levels such as bladder, leukoplakia, lung, prostate, esophageal, and colorectal cancer (Dammann et al., 2014). Studies with molecular biomarker analysis or targets downstream of PAKs were included. Studies without data were excluded from analysis. Clinical analyses of overall response rate, adverse events, as well as molecular analysis of biomarkers were performed on pooled data from pioglitazone or metformin treated patients.
RESULTS

PAK signaling is associated with diabetes and cancer

Previous studies have provided a role for PAK in both diabetes and cancer, however a clear depiction of the signaling pathways involved in both diseases has not been performed. Systematic review of the literature demonstrated 14 PAK signaling pathways, either upstream or downstream of PAK, and 11 or 78% of these pathways were also associated with glucose homeostasis (Table 1 & 2; Supplementary Figure 1 & 2). All of the PAK signaling pathways which were associated with glucose homeostasis were also associated with malignant inflammatory, proliferative, survival, and angiogenic signaling which occurs in diseases such as cancer (Table 3 and Supplementary Figure 3).

PAK interacting partners are associated with the pathogenesis of T2DM

The pathogenesis of T2DM was previously linked to obesity, insulin resistance, and molecular alterations of pancreatic beta cells (McCarthy, 2010). PAK signaling mediates pathways involved in glucose homeostasis (Table 2); but whether PAK or its partners are actually involved in the pathogenesis of T2DM is unknown. We identified protein-protein interactions and prospective interacting partners in the pathogenesis of T2DM by screening PAKs 1-6 and previously identified markers of reduced beta cell mass (Figure 1A), dysfunction (Figure 1B), and obesity and insulin resistance (Figure 1C), using the online STRING database (Szklarczyk et al., 2015).

Thirty total PAK interacting partners were found to involve reduced Beta-cell mass (10), Beta-cell dysfunction (10), and obesity and insulin resistance (10) (Figure 1D and Supplementary Table 1). Signaling pathways associated with PAK
interacting partners included cell cycle control, receptor tyrosine kinases (RTK), G-proteins, and Wnt signaling (Figure 1E). Reduction in Beta-cell mass was exclusively linked to eight-interacting partners involving cell cycle control. Beta-cell dysfunction was found to be associated with four-interacting partners linked to Wnt signaling, and six-interacting partners involving RTKs were associated with obesity/insulin resistance (Figure 1E). G-protein signaling involved two-interacting partners involved in reduced Beta-cell mass, five-partners in Beta-cell dysfunction, and four-partners in obesity/insulin resistance (Figure 1E). These data suggest PAK interacting partners are associated with and utilize the cell cycle, Wnt, RTK, and G-protein signaling in the pathogenesis of T2DM.

**Upstream and downstream PAK signaling pathways are utilized by anti-diabetic drugs pioglitazone and metformin**

We found that PAK interacting partners were involved in the pathogenesis of T2DM, however the role of PAK signaling in T2DM remained elusive. We investigated this by analyzing the effect of anti-diabetic drugs metformin (met), glyburide (gly), and glitazones (glit) on PAK signaling pathways (Table 4). Overall, glit and met each altered ten PAK signaling pathways whereas gly altered three (Figure 2A). Further analysis revealed all three anti-diabetic drugs altered pathways upstream and downstream of PAK (Figure 2B). Glit exerted the most profound effect on downstream PAK signaling pathways [glit=9 vs met=7 vs gly=3]. Met altered more pathways upstream of PAK [met=5 vs glit=3 vs gly=1] (Figure 2B).

Malignant PAK signaling is involved in disease pathogenesis. To further understand the role in which met, gly, and glit interfere with PAK signaling in T2DM, we asked if any of these drugs activate or inhibit inflammatory,
proliferative, survival, or angiogenic pathways upstream and downstream of PAK (Figure 2C-F). Differential pathway analysis revealed met and glit consistently altered more pathways than gly (Fig 2C-F). Glit inhibited more pathways downstream of PAK than met in [inflammation: glit=7 vs met=4], [proliferation: glit=8 vs met=4], [survival: glit=8 vs met=4], [angiogenesis: glit=8 vs met=4] (Figure 2C-F). However, met activated more pathways than glit which are exaggerated upstream of PAK and involved in inflammation [met=4 vs glit=2;], proliferation [met=2 vs glit=0], survival [met=3 vs glit=1], and angiogenesis [met=3 vs glit=1] (Figure 2C-F). These data suggest that antidiabetic drugs alter PAK signaling. Metformin activates pathways upstream and glitazones inhibit pathways downstream of PAK.

**Pioglitazone and metformin have therapeutic limitations in cancer patients with PAK overexpression**

Previous clinical trials have attempted to establish whether met and pio therapeutically have chemopreventive activity in humans, but the results remain highly ambiguous. We showed that met and pio altered PAK signaling upstream and downstream of PAK, and asked whether the chemopreventive nature of these drugs was found in diseases with PAK overexpression. To investigate this, we analyzed clinical studies in diseases known to overexpress PAK in which met or pio treatment was tested (Table 5).

Initially, we screened hundreds of studies on ClinicalTrials.gov for diseases with PAK overexpression and either met or pio treatment. We found one observational-prospective cohort and six interventional studies (three single arm and three randomized double blind) with these criteria (Table 5 and Figure 3A).
We investigated the effect of anti-diabetic therapy in patients with PAK overexpression by calculating an overall clinical response rate (OCRR), which was defined as the total number of patients who had ≥ 50% decrease in the sum of all their lesions post-treatment. The OCRR in pio-treated patients was 53% (Figure 3B); 28/53 patients responded and 25/53 did not. The corresponding data for met were unavailable. To further evaluate patient outcome, we calculated the number of serious or other adverse events after pio and met treatment (Figure 3B). Comparison of pio and met treatment revealed a higher percentage of serious [pio: 3/53 = 5.7% vs met: 3/91 = 3.3%] and other [pio: 42/53 = 79% vs met: 68/91 = 74.7%] adverse events upon treatment with pio (Figure 3B). Additionally, in comparison to patients treated with met, a fewer percentage of pio-treated patients were without adverse effects [pio: 11/53 = 21% vs met: 25/91 = 27.4%] (Figure 3B). Pioglitazone associated events included edema (15%), oral pain (13%), and hypertension (7.5%), while those associated with metformin were gastrointestinal symptoms like constipation (7%), diarrhea (23%), and nausea (13%). These data suggest that patients with tumors which are known to have PAK overexpression have a good clinical response to pio; and both pio and met treatment are associated with a high rate of mostly mild adverse events.

Pioglitazone and metformin alter biomarkers downstream of PAK in human disease

We found that patients which have diseases overexpressing PAK responded to pio, however it was unclear whether pio or met actually interfered with PAK signaling. We therefore analyzed biomarkers downstream of PAK signaling pathway involved in apoptosis, cell cycle, PI3K/mTOR, and PPARγ (Table 6).
We calculated the percent change in biomarker expression in pio and met treated patients to visualize the molecular effect on PAK signaling (Figure 4). Molecular analysis revealed that pio treatment decreased expression levels of multiple biomarkers including, apoptotic marker BCL2, and cell cycle markers CyclinD1 and Ki67, respectively. Pio treatment increased total and cytoplasmic PPARγ levels by 50% and 8%, however it decreased nuclear expression by 32% (Figure 4). Met treatment resulted in an increase in cell cycle biomarker Ki67, and did not alter expression levels of PI3K/mTOR markers PS6K1 or PS65Ser235 (Figure 4). These data indicate that pio inhibited pathways involved in apoptosis and cell cycle regulation whereas met stimulated or did not alter these pathways in patients which potentially have aberrant PAK signaling.
DISCUSSION

Type 2 diabetes is a major cause of disability and death worldwide, and in addition to its multiple disease specific complications, it also carries and increased cancer risk, such as colon cancer (Chang & Yang, 2016). P-21 activated kinases are serine-threonine kinases, which influence multiple cell functions from normal cell signaling to cancer (Kumar et al., 2016). Physiological PAK signaling is regulated and implicated in the maintenance of cellular homeostasis, however as the extracellular microenvironment or PAK expression in disease changes, physiological signaling becomes pathological (Figure 5). Here we reviewed physiological PAK signaling and found it is highly associated with glucose homeostasis, although PAKs role in the pathogenesis of T2DM, and cancer in diabetes patients is largely unknown.

PAK overexpression is associated with disease severity; however, in diabetes, Ahn et al. previously reported a reduction in PAK1 expression in beta cells of the pancreas (Ahn et al., 2016). Here we emphasized the importance of PAK signaling (kinase activity) versus total expression levels, as both chronic inflammation and hyperinsulinemia associated with T2DM (Chang & Yang, 2016) (Donath & Shoelson, 2011), likely leads to pancreatic and peripheral PAK activation. Additionally, six different PAKs have been characterized; therefore signaling from other PAKs may compensate for reduction in PAK1 expression. We identified novel PAK interacting partners associated with the pathogenesis of T2DM, involving reduced beta cell mass and dysfunction, and obesity-insulin resistance (Figure 1). Interestingly, we identified PAK interacting partner IQGAP1 was involved in all three pathways (Figure 1D). IQGAP1 acts as a
Dammann et al., 2018

molecular scaffold for small Rho-GTPase activation of PAKs (R. Li et al., 1999), which further implicates the importance of PAK kinase activation in T2DM.

The initial pathogenesis of T2DM involves beta cell expansion to compensate for hyperglycemia, which eventually may lead to reduced beta cell mass (McCarthy, 2010). Here, PAK interacting partners involved with reduced beta cell mass and dysfunction were specifically associated with cell cycle regulation and Wnt signaling (Figure 1D). It is possible that in early diabetes, hyperglycemia promotes cell cycle progression via PAK- Beta-catenin signaling (Park et al., 2012). Over time, PAK driven proliferation and inflammation may lead to oxidative stress (Reuter et al., 2010) and therefore contribute to beta cell dysfunction and subsequent reduction in beta cell mass (Drews et al., 2010).

Interestingly, in oxidative stress nuclear Beta-catenin was reported to associate with FOXO transcription factors (Essers et al., 2005), which could potentially modulate PAK expression levels (Mazumdar & Kumar, 2003) in line with reports from Ahn et al. Another explanation for PAK’s role here may involve an interaction with tumor suppressor p53 (Figure 1A and D). Interestingly, activation of both p53 and MDM2, a p53 ubiquitin ligase, was reported in T2DM (Xiaomu et al., 2016), and a PAK–MDM2 interaction was previously described (T. Liu et al., 2013). Others provided a role for PAK upstream of p53 (Murray et al., 2010) (Park et al., 2009), thus PAK signaling in T2DM may activate p53 directly, or indirectly via MDM2 or through a cell stress pathway such as oxidative stress or stress associated MAPK like p38/JNK (Shi et al., 2014) thereby inducing apoptosis, impeding cell cycle progression, and subsequently reducing beta cell mass. Additional PAK interacting partners associated with pathways involving obesity-insulin resistance involved targets in RTK signaling, which is likely a
consequence of multiple growth factors, and the chronic inflammatory state associated with obesity in T2DM (Donath & Shoelson, 2011).

We sought to illuminate the role of PAK signaling in T2DM by analyzing the effect that three well-known classes of anti-diabetic drugs had on PAK signaling (Figure 2). Biguanide (metformin), sulfonylurea (glyburide), and glitazone (pioglitazone) all interfered with signaling upstream and downstream of PAKs, and this effect was more apparent with metformin (upstream) and pioglitazone (downstream) of PAKs (Figure 2). Both metformin and pioglitazone mediated inflammatory, proliferative, survival, and angiogenic pathways associated with PAKs (Table 4 and Figure 2).

Considering the role of PAK signaling in the initiation of disease (Kumar et al., 2016), and that PAK signaling appears to be activated in T2DM (Table 2), and inactivated by anti-diabetic drugs (Figure 2), we investigated whether metformin or pioglitazone were beneficial in patients with PAK-overexpressing diseases including oral cancer (Parvathy et al., 2016), non small cell lung cancer (Y. Liu et al., 2016), prostate (T. Liu et al., 2013), esophageal cancer (Gan et al., 2015), bladder (Ito et al., 2007), and colorectal cancer (Song, Wang, Zheng, Yang, & Xu, 2015). Pre-clinical studies investigating the chemopreventive effects of these drugs seemed promising (Girnun et al., 2002) (Grommes et al., 2013) (Burotto & Szabo, 2014), however results from human studies remain highly ambiguous (Lewis et al., 2015) and a more complete analysis of this data would allocate whether inhibition of PAK by metformin or pioglitazone is a promising for chemoprevention.

We performed a systematic review of several studies (Table 5) involving metformin and pioglitazone use in cancer patients to investigate whether these
treatments are potential candidates to target PAK signaling (Figure 3). Our analysis provided that pioglitazone treated patients with PAK dependent diseases had a clinical response rate of 53% (Figure 3B). It was unfortunate that data needed to calculate response rate to metformin were not available for analysis. Both pioglitazone and metformin treatment resulted in relatively few serious adverse events. However, the overwhelming majority of patients, nearly 80% of pioglitazone and 75% metformin treatment patients had mild adverse events (Figure 3B). The high number of pioglitazone associated events such as edema, and hypertension were likely due to the advanced treatment regimen used in these patients, equivalent to 45mg/day, versus the standard care at a dose equivalent to 15mg/day (Majima et al., 2006). Other studies have indicated fewer adverse events at lower doses equivalent to 7.5mg/day (Majima et al., 2006). Although gastrointestinal symptoms like constipation, diarrhea, and nausea are common side effect of metformin (Bosi, 2009), so many adverse events were unexpected and likely not attributable to dose, as patients received a standard of care equivalent of 2000mg/day. Given that metformin is the gold standard in treatment of T2DM (Bosi, 2009), the frequency of adverse events is concerning. Although not life threatening, adverse events are a serious therapeutic limitation and concern for future chemopreventive studies, as long-term patient compliance will dramatically decline if quality of life is decreased by therapy. In addition to our analysis, ten-year treatment of pioglitazone in diabetic patients was associated with increased prostate and pancreatic cancer risk, (Lewis et al., 2015), making its long-term use in chemoprevention questionable.
We verified whether PAK signaling was even affected in these patients by pioglitazone or metformin at the molecular level (Table 6 and Figure 4), and found that pioglitazone but not metformin decreased PAK signaling. This data was in line with our analysis of signaling pathways affected by these drugs (Table 4), in that metformin induced signaling upstream of PAKs while pioglitazone rather reduced downstream signaling (Figure 2). Here pioglitazone reduced markers of proliferation including cyclinD1 and Ki67, while metformin had the opposite effect on proliferation. Long-term treatment with metformin in diabetic patients may therefore induce, not inhibit long-term cancer risk, however future studies would need to investigate this further.

Considering our clinical and molecular analysis of PAK biomarkers in pioglitazone treated patients, future studies should investigate the long term side effects associated with its treatment and whether PAK signaling in disease can be impeded early on, to block malignant transformation (Kumar et al., 2016).

This was the first study to provide a mechanistic explanation of how anti-diabetics may target PAK signaling for their potential use for chemoprevention in patients with T2DM, albeit our signaling pathway analysis included multiple pre-clinical studies, which were not of high significance. Ideally, a thorough signaling pathway analysis should use PAK1 and p-PAK1 as biomarkers in multiple human studies, however this data was unavailable for analysis due to the limitations of the current literature in regard to our highly specific question (Dammann et al., 2015). Considering the few patients which were analyzed here, the accuracy of this data is supported by its correlation between literature reports (Table 4 and Figure 2) and our analysis of PAK signaling pathways from human studies (Table 6 and Figure 4). Future studies with more patients and
additional readouts of known PAK targets would provide a more clear analysis of whether chemoprevention in diabetes with pioglitazone is feasible long term.

**Future directions**

Although, not associated with diabetes, other studies of chemoprevention have shown that anti-inflammatory drugs like (aspirin in colorectal cancer), and (mesalamine in colitis associated cancer), reduce cancer-associated risk (Drew et al., 2016) (Lyakhovich & Gasche, 2010). Mesalamine, the first line treatment for chronic inflammation in ulcerative colitis (Karagozian & Burakoff, 2007), was recently established as a PAK1 inhibitor (Khare et al., 2013), and others have shown it is a PPAR-gamma ligand (Rousseaux et al., 2005); and both of these mechanisms are in line with glitazones (Dammann et al., 2015). Therefore, future directions in chemoprevention in diabetes should analyze PAK expression/phosphorylation upon glitazone treatment and see if the effects are similar to those of mesalamine in impeding chronic inflammation in colitis associated colon cancer.

**Concluding remarks**

Anti-diabetics like pioglitazone rather than metformin should be utilized as a platform for further understanding the role of PAKs as a chemopreventive target in diabetes; however, before this is possible, future studies must standardize doses specific to the associated disease in order to modulate PAK signaling appropriately and minimize adverse effects. Ideal chemoprevention, like mesalamine, will block inflammation, and impede aberrant PAK signaling without altering cellular homeostasis.
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Dammann et al., 2018


