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# TPCK inhibits AGC kinases by direct activation loop adduction at phenylalanine-directed cysteine residues

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ABSTRACT

**N-alpha-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) has anti-tumorigenic properties, but its direct cellular targets are unknown. Previously, we showed TPCK inhibited the PDKI-dependent AGC kinases RSK, Akt and S6K1 without inhibiting PKA, ERK1/2, PI3K, and PDK1 itself. Here we show TPCK-inhibition of the RSK-related kinases MSK1 and 2, which can be activated independently of PDK1. Mass spectrometry analysis of RSK1, Aktl, S6K1 and MSK1 immunopurified from TPCK-treated cells identified TPCK adducts on cysteines located in conserved activation loop Phenylalanine–Cysteine (Phe–Cys) motifs. Mutational analysis of the Phe–Cys residues conferred partial TPCK resistance. These studies elucidate a primary mechanism by which TPCK inhibits several AGC kinases, inviting consideration of TPCK-like compounds in chemotherapy given their potential for broad control of cellular growth, proliferation and survival.**


## 1. Introduction

The ability of cells to perceive and appropriately respond to their microenvironments is crucial to essentially all cellular processes. Protein kinases are key modulators and drivers of these processes and therefore have become attractive targets for drug discovery. The functionally diverse but structurally conserved family of kinases termed the AGC kinases [1] (as they contain relatives of protein kinase A (PKA), protein kinase G (PKG) and protein kinase C (PKC)) includes isoforms of Akt (protein kinase B (PKB)) [2], p70 ribosomal S6 kinase (S6K) [3], p90 ribosomal S6 kinase (RSK) [4], mitogen- and stress-activated protein kinase (MSK) [5], and serum- and glucocorticoid-induced protein kinase (SGK) [6]. Despite divergent regulation, these kinases are all activated by the common kinase regulatory mechanism of phosphorylation at the autoinhibitory activation loop [7].

3-Phosphoinositide-dependent kinase 1 (PDK1) has been shown to phosphorylate the activation loops of several AGC kinase family members [1,8]. Additionally PDK1 phosphorylates its own activation loop [9]. Despite the emergence of PDK1 as a critical regulator of multiple AGC kinases with oncogenic potential, few studies have evaluated PDK1 as a potential target for cancer therapy. However, PDK1's value as a target for anticancer therapy is certainly under consideration [10–12]. Previously we reported that TPCK disrupts PDK1 signaling to Akt, S6K1, and RSK [13]. However, the inhibitory mechanism was unclear given TPCK did not directly affect the phospshotransferase activity of PDK1. Here we investigated the inhibitory effects of TPCK toward the non-PDK1 dependent kinases MSK1 and MSK2 and found that they were TPCK sensitive. We next used mass spectrometry to analyze AGC kinases immunoprecipitated from TPCK-treated cells and found that TPCK-inhibited kinases showed direct TPCK adduction at phenylalanine-directed cysteine (Phe–Cys) residues in their activation loops. Mutation of the conserved cysteine or phenylalanine led to the generation of kinases partially TPCK-resistant. This study thus describes a primary mechanism by which TPCK inhibits specific AGC kinases and describes novel TPCK resistant AGC kinase alleles. Furthermore we discuss the potential productivity of developing inhibitors such as TPCK that could simultaneously target conserved Phe–Cys motifs in several AGC kinases. Such a drug could dampen multiple signaling arms of a cell in a state of hyperactivity and thereby reduce the chances of such cells developing resistance, a major thorn in the side of targeted cancer therapies in our emerging era of personalized medicine.

*Abbreviations:* TPCK, N-alpha-tosyl-L-phenylalanyl chloromethyl ketone; PDK1, 3-phosphoinositide dependent protein kinase-1; AGC, related to protein kinase A, protein kinase G and protein kinase C; RSK, ribosomal S6 kinase; S6K, S6 kinase; PKA, protein kinase A; ERK, extracellular signal regulated kinase; PI3K, phosphatidylinositol-3 kinase; MSK, mitogen and stress-activated protein kinase

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## 2. Materials and methods

### 2.1. Plasmids

The plasmids encoding triple hemagglutinin ($HA_3$)-tagged RSK1 [14] and HA-S6K1 [15] have been described. Bacterial expression plasmids encoding GST-S6 (rat) Lys218-Lys249 [16], GST-BAD [16], GST-RSK1-D1 K/R have been described. Myc-tagged PDK1 in pCDNA3 was a gift of P. Hawkins. HA-Akt in pCMV6 was a gift of P. Tsichlis. Flag-tagged MSK1 was a gift of R. Janknecht. The cloning of $HA_3$-MSK2 was as follows: EST clone AA576979 from Genome Systems was used to generate a $^{32}$P-labeled probe that was used to screen $1.7 \times 10^6$ plaques from a human T-cell cDNA library in Lambda ZAPII (Stratagene) at 45,000 plaques per 15 cm dish. The primers used to generate the probe were 5′-GCTCAGAGCTG-GATGTGG-3′ (sense) and 5′-TCGGCGTACAGGATGTTC-3′ (anti-sense). Twelve positive clones were ultimately identified and the longest clone contained the start codon and extended ~1,9000 bp and included an internal NotI site. The 3′-end of MSK2 from the internal NotI site and extending past the stop codon ~600 bp was obtained from Genome Systems EST clone AI831613. The full length sequence was assembled first in pBluescript SK-. The 5′-untranslated region was removed while inserting a BglII site at the 5′-end using PCR amplification from the initiating codon and past an internal EcoRI site. The PCR primers were 5′-AGAGATCTATGGGGGAC-GAGGACGAC-3′ (sense) and 5′- GGAATTCTTAGGAGGGGGGCAGGG GGCCGTT-3′ (anti-sense). The 5′-BglII, 3′-EcoRI fragment was subcloned into the BamHI, EcoRI sites of pKH3 in frame with the triple HA-tag (at the 5′-end). The remainder of MSK2 (from the internal EcoRI site, past the stop codon and including the 3′ untranslated region to an EcoRI site in pBluescript) was subcloned into the pKH3 that already contained the BglII-EcoRI fragment of MSK2 described above, using the EcoRI fragment excised from pBluescript-MSK2. The insert was sequenced and we found all nucleotides to be identical to those published under GenBank accession AJ010119 except for a T2237C (numbering is from AJ010119) substitution that did not change the amino acid sequence.

### 2.2. Mammalian cell culture, transfection and lysis

E1 A-transformed Human embryonic kidney (HEK 293E) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin and streptomycin. HEK293E cells were transfected using calcium phosphate precipitation. Twenty four hours post-transfection, cells were starved in DMEM containing 20 mM HEPES for 16–18 h prior to stimulation and as indicated in the figure legends. After stimulation, cells were washed twice with cold phosphate-buffered saline (PBS) and then lysed in 10 mM KPO4, 1 mM EDTA, 10 mM $MgCl_2$, 50 mM $\beta$-glycerophosphate, 5 mM EGTA, 0.5% Nonidet P-40, 0.1% Brij 35, 0.1% deoxycholic acid, 1 mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml of pepstatin A. The cell extracts generated were centrifuged at 14,000 rpm for 10 min to remove cell debris and the clarified supernatant was used for immunoprecipitations, immunoblotting or protein kinase assays.

### 2.3. Immunoprecipitations, kinase assays and immunoblotting

Cell lysates were incubated with endogenous or anti-epitope tag antibodies for 2 h and then with 20 μl of a 1:1 mixture of protein G-sepharose and protein A-sepharose beads for an additional hour at 4 °C. Beads were washed three times with lysis buffer and the immunoprecipitates were used for kinase assays or immunoblotting. Kinase assays for RSK1-4, MSK1-2 and S6K1 were performed using GST-S6 as a substrate. Kinase assays for Akt used GST-BAD as a substrate. Kinase assays for PDK1 used RSK-D2 K/R as a substrate. Kinase assays were allowed to proceed for 10 min. at 30 °C prior to stopping the reaction with sample buffer as described previously [13]. The reaction products were subjected to SDS–PAGE and dried gels were exposed to X-ray film. For immunoblots, immune complexes or cell lysates were subjected to SDS–PAGE and transferred to nitrocellulose membranes. Blocking, primary and secondary antibody incubations of immunoblots were performed in TBST (10 mM Tris, pH 7.4, 150 mM NaCl and 0.1% Tween 20) supplemented with 5% (wt/vol) dry skim milk powder. Horseradish peroxidase-conjugated donkey anti-rabbit and anti-mouse antibodies were used to facilitate detection by enhanced chemiluminescence (ECL) and exposure to X-ray film.

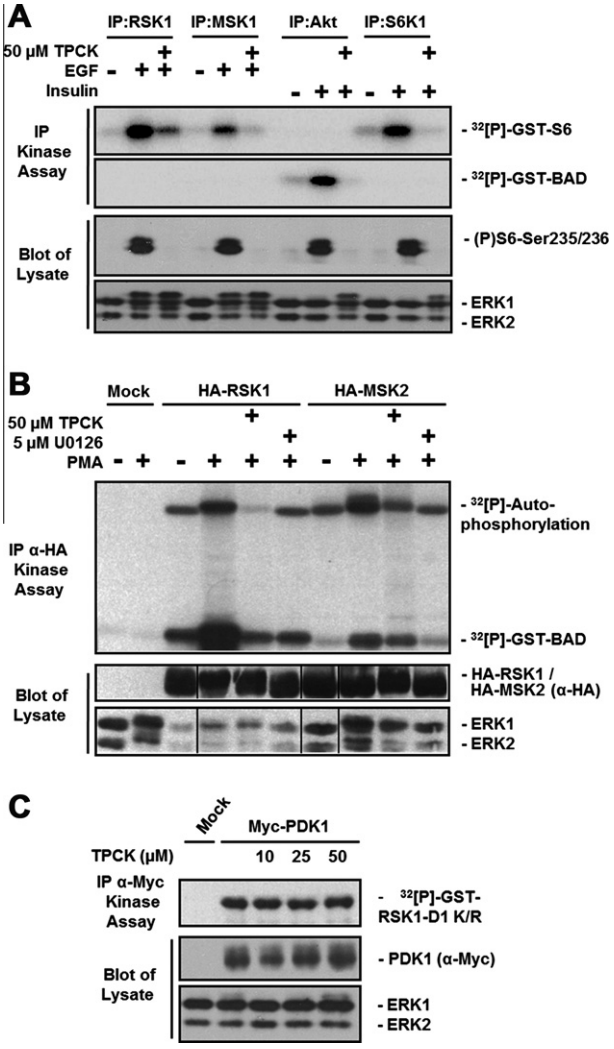


**Fig. 1.** TPCK inhibits PDK1-dependent and PDK1-independent AGC kinases (A) TPCK inhibits the PDK1-dependent kinases RSK, Akt and S6K1 (A and B) as well as the non-PDK1-dependent AGC kinase MSK1 and MSK2 (C) without inhibiting PDK1 kinase activity. HEK293E cells were serum-starved for 18 h, and treated with either EGF (50 ng/ml) for 10 min. or Insulin (100 nM) for 30 min. (as indicated) with or without pretreatment of cells with TPCK for 30 min. as indicated. Endogenous kinases (RSK1, MSK1, Akt1 and S6K1) or exogenous kinases (HA-MSK2 and Myc-PDK1) were immunoprecipitated with their respective antibodies. These immunoprecipitates were incubated with either GST-S6, GST-Bad or GST-RSK-D2 K/R (as indicated) in a kinase reaction containing [$\gamma - ^{32}$P] ATP for 10 min. The samples were subjected to SDS–PAGE, and the gel was autoradiographed. The lysates were immunoblotted for pAkt, pS6, ERK1/2, HA or Myc as indicated.