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Functional proteomics strategy for validation of protein kinase inhibitors reveals new targets for a TBB-derived inhibitor of protein kinase CK2☆

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ABSTRACT

CK2 is a constitutively active protein kinase with key regulatory roles in many cellular signaling events which has been implicated in several human diseases. To investigate its roles in biological events and potential as a therapeutic target, several potent CK2 inhibitors have been developed including TBB and its derivatives that have been employed in many studies. Despite the utility of these compounds, a precise understanding of their mode of action within cells remains incomplete. In fact, cells are typically treated with inhibitor concentrations (>5 μM) that are orders of magnitude higher than their in vitro inhibitory constants (<0.05 μM). Accordingly, we hypothesized that CK2 inhibitors could have off-target effects that are not recognized when inhibitors are profiled using panels of recombinant protein kinases. To address this issue, we combined structural modeling with inhibitor-affinity purification and proteomics to test the specificity of derivatives of TBB using whole cell lysates of HeLa cells. While these studies confirmed that CK2 does bind to the immobilized inhibitor, several other abundant ATP/GTP-binding proteins were also identified and confirmed. These results suggest that highly abundant nucleotide binding proteins may limit the bioavailability of the free inhibitor and interactions with CK2 in the cellular environment.
This article is part of a Special Issue entitled: From protein structures to clinical applications.

1. Introduction

Protein phosphorylation is a reversible modification that plays prominent roles in the transmission of regulatory information within cells. The universal nature of phosphorylation is emphasized by the fact that more than 500 protein kinases are encoded within the human genome [1,2]. Since perturbations in protein kinase-mediated signaling pathways have been implicated in many diseases, protein kinases have emerged as attractive targets for therapy [3]. Proof-of-principle for the utility of protein kinase inhibitors as therapeutic agents comes from the successful example of Gleevec (imatinib) that is used to treat Philadelphia chromosome-positive chronic myeloid leukemia and some other cancers [4–8].

Protein kinase CK2 (two isoforms designated as CK2alpha and CK2alpha′ in mammals) is a constitutively active protein kinase [9] with an ever-expanding list of interacting partners [10,11] and substrates [12]. CK2 levels are elevated in many

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forms of cancer including prostate [13], mammary gland [14], lung [15], kidney [16] and head and neck [17]. Moreover, CK2 displays oncogenic activity in mouse models [14] and pharmacological inhibition or knockdown of CK2 results in apoptosis in a number of cancer cell types [18–22]. These observations have spurred a number of efforts to develop CK2 inhibitors including compounds that are now in human clinical trials [23,24]. While a small proportion of these inhibitors can bind CK2 allosterically [25], the majority of CK2 inhibitors are structurally similar to ATP or GTP and target its catalytic pocket [26,27]. As is the case with many protein kinase inhibitors, the selectivity of newly developed CK2 inhibitors is generally evaluated using a panel of recombinant protein kinases (often <70 of 500 kinases) for in vitro phosphorylation assays [26,28–30]. Although information from these panels of recombinant enzymes provides extremely valuable insights, they fail to provide a comprehensive view of cellular targets for the inhibitor in the entire proteome [30–34]. In the absence of this information, many questions remain regarding the interpretation of experiments that are performed with these compounds. In a similar respect, a comprehensive evaluation of inhibitor targets would undoubtedly be an important consideration when contemplating potential clinical applications [35].

To overcome this limitation, we employed a functional proteomics strategy using an immobilized CK2 inhibitor to comprehensively evaluate inhibitor targets for a derivative of 4,5,6,7-tetrabromo-1*H*-benzotriazole (TBB), one of the most widely used commercially available CK2 inhibitors. For these studies, we immobilized a mixture of 5-methyl-TBB derivatives (collectively designated 5-methyl-TBB; Supplementary Fig. 1) on Sepharose to generate an affinity matrix to capture proteins from HeLa cell lysates. Our studies confirmed that CK2 does indeed interact with 5-methyl-TBB. However, we also identified several other more abundant off-target proteins that were captured by the immobilized inhibitor. A common feature of these off-targets is their nucleotide binding. Our findings may illuminate at least in part why a CK2 inhibitor with a K_i in the nanomolar range must typically be used in cell culture at micromolar concentrations [28,32,33,36–39] to effectively inhibit endogenous CK2.

2. Materials and methods

2.1. Reagents, cell culture and antibodies

The CK2 inhibitor 4,5,6,7-tetrabromo-1*H*-benzotriazole (TBB) and other reagents used in the experiments were from Sigma unless indicated otherwise. Epoxy-activated Sepharose 6B (GE Healthcare) was used as the affinity matrix in all experiments. HeLa (Tet-Off®, Clontech) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 units/mL penicillin (Invitrogen) at 37 °C with 5% CO_2 in 15-cm dishes (Falcon). The antibodies for immunoblotting the endogenous CK2alpha, alpha′ or beta were used as previously described [32,33,40,41]. Anti-heat shock protein 90 (HSP90alpha/beta, 1:1000, Santa Cruz), anti-MEK1/2 (1:1000, Cell signaling) and anti-glyceraldehyde-3-phosphate dehydrogenase, clone 6C5 (GAPDH, 1:1000, Millipore) antibodies were all obtained from commercial suppliers and anti-tubulin beta monoclonal antibody was a generous gift of Dr. L. Dagnino (University of Western Ontario, Dept. of Physiology and Pharmacology). All secondary Infrared IRDye®-labeled antibodies (LiCor) (1:10,000 dilution in PBS containing 1% BSA and 0.1% Tween 20) were from LiCor.

2.2. Synthesis of TBB derivative and immobilization to Epoxy-activated Sepharose 6B

The starting material (5-methyl-4,6,7-tribromo-1*H*-benzotriazole) was synthesized as previously reported [42]. Next, 3-(4,6,7-tribromo-5-methyl-1*H*-benzotriazol-1-yl)propan-1-amine and 3-(4,6,7-tribromo-5-methyl-2*H*-benzotriazol-2-yl)propan-1-amine were synthesized by mixing 5-methyl-4,6,7-tribromo-1*H*-benzotriazole (27 mM) in pyridine (10 mL) with Triton B (500 μL) and allylamine (27 mM) under constant stirring. The reaction mixture was incubated for 5 h at 80 °C under continuous stirring, cooled and then diethyl ether (20 mL) was added. The precipitate was collected by filtration and the structure was confirmed by mass spectrometry (Waters Q-Tof Premier in the Mass Spectrometry Lab, IBB PAS). Crystallization from ethyl acetate yielded a mixture of 3-(4,6,7-tribromo-5-methyl-1*H*-benzotriazol-1-yl)propan-1-amine and 3-(4,6,7-tribromo-5-methyl-2*H*-benzotriazol-2-yl)propan-1-amine (1.49 g) that was coupled in dioxane/water (1:1) to Epoxy-activated Sepharose 6B (5 g), and afterward blocked with 1 M ethanolamine following the manufacturer's recommendation. Prior to affinity-chromatography, the remaining active groups of control Sepharose or 5-methyl-TBB Sepharose were blocked for 16 h with 1 M ethanolamine pH 8.0. Matrices were then washed three times with alternating pH buffers (0.1 M Tris–HCl, 0.5 M NaCl, pH 8.5; 0.1 M acetate buffer, 0.5 M NaCl, pH 4.0). The reconstituted matrixes were stored in PBS containing 0.05% NaN_3, pH 7.2 in the dark at 4 °C.

2.3. In vitro CK2 inhibition assay

Effect of 5-methyl-TBB Sepharose on CK2alpha was evaluated using the CK2 peptide substrate (20 μM RRRDDDSDDD, Biaffin GmbH & Co KG, Germany). Kinase reactions (50 μL) contained CK2alpha (2 ng/μL, KinaseDetect ApS, Denmark) in Tris–HCl pH 7.5 (20 mM), $MgCl_2$ (20 mM), sodium β-glycerol phosphate (380 μM), EGTA (80 μM), DTT (15 μM), with 0.86 mg of control or 5-methyl-TBB Sepharose. Following incubation for 20 min at room temperature, γ-32P-ATP (10 μM, 70–200 cpm $pmol^{-1}$) was added and the reaction mixtures were further incubated for 20 min at 30 °C. Phosphorylation reactions (20 μL) were spotted onto P81 papers, washed three times with 0.6% o-phosphoric acid and then air dried. The incorporation of γ-32P-ATP was quantified by liquid scintillation counting (Perkin Elmer TriCarb 2910TR scintillation counter).

2.4. Structural modeling

A structural model of 3-(4,6,7-tribromo-5-methyl-1*H*-benzotriazol-1-yl)propan-1-amine was generated using Avogadro v. 1.0.3 (http://avogadro.openmolecules.net/) open source software based on the chemical structure in Fig. 1A. For this model, the structure of the 3-(4,6,7-tribromo-5-methyl-1*H*-