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Development of novel molecular probes of the Rio1 atypical protein kinase ☆

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ABSTRACT

The RIO kinases are essential protein factors required for the synthesis of new ribosomes in eukaryotes. Conserved in archaeal organisms as well, RIO kinases are among the most ancient of protein kinases. Their exact molecular mechanisms are under investigation and progress of this research would be significantly improved with the availability of suitable molecular probes that selectively block RIO kinases. RIO kinases contain a canonical eukaryotic protein kinase fold, but also display several unusual structural features that potentially create opportunity for the design of selective inhibitors. In an attempt to identify structural leads to target the RIO kinases, a series of pyridine caffeic acid benzyl amides (CABA) were tested for their ability to inhibit the autophosphorylation activity of *Archeaoglobus fulgidus* Rio1 (*Af*Rio1). Screening of a small library of CABA molecules resulted in the identification of four compounds that measurably inhibited AfRio1 activity. Additional biochemical characterization of binding and inhibition activity of these compounds demonstrated an ATP competitive inhibition mode, and allowed identification of the functional groups that result in the highest binding affinity. In addition, docking of the compound to the structure of Rio1 and determination of the X-ray crystal structure of a model compound (WP1086) containing the desired functional groups allowed detailed analysis of the interactions between these compounds and the enzyme. Furthermore, the X-ray crystal structure demonstrated that these compounds stabilize an inactive form of the enzyme. Taken together, these results provide an important step in identification of a scaffold for the design of selective molecular probes to study molecular mechanisms of Rio1 kinases *in vitro* and *in vivo*. In addition, it provides a rationale for the future design of potent inhibitors with drug-like properties targeting an inactive form of the enzyme. This article is part of a Special Issue entitled: Inhibitors of Protein Kinases (2012).

1. Introduction

Protein kinases comprise one of the largest protein superfamilies. They play crucial roles in fundamental cellular processes, including metabolism, cell cycle, cell division and differentiation, and apoptosis. The importance of protein phosphorylation in eukaryotic signaling is reflected in the fact that protein kinase domains are found in 1.7% of all human genes (518 genes, [1]). About half of them are linked to oncogenesis and other major diseases [2,3]. Therefore kinases are attractive molecular targets for therapeutic intervention.

Comprehensive analysis of the human genome performed by Manning and coworkers (2002) resulted in the annotation of 13 atypical protein kinase families (aPKs) [1]. Atypical protein kinases families are designated as such due to limited sequence homology with canonical eukaryotic protein kinases (ePKs) but demonstrated protein kinase activity among its members. The RIO family (named after Rio1 - *right open reading frame 1*) is one such family, characterized by proteins containing the conserved kinase-like RIO domain [4]. Four RIO subfamilies thus far have been identified: Rio1, Rio2, Rio3 and RioB (in some eubacteria) [5,6]. Rio1 and Rio2 are strongly conserved from Archaea to man, whereas Rio3 is present only in metazoa [5]. In yeast and humans, they are involved in processes critical for cellular proliferation, namely cell cycle progression, ribosome biogenesis and chromosome maintenance. Yeast studies reported that the catalytic activity of Rio1 and Rio2 kinases are required for proper processing of 20S pre-rRNA

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to mature 18S rRNA — the RNA component of the 40S ribosome subunit. The yeast Rio1 kinase is also required for proper cell cycle progression [4,7,8]. Deletion or depletion of Rio1 or Rio2 results in accumulation of intermediate metabolite rRNA, 20S rRNA, in yeast and human cells [7,9].

The X-ray crystal structures of Rio1 and Rio2 from *Archaeoglobus fulgidus* have been previously determined [10–12]. The RIO domain is structurally homologous to canonical eukaryotic protein kinase domain but is a variant by deletion of loops utilized for peptide substrate binding and kinase activation. Canonical protein kinase domains consist of two parts or "lobes" connected by a flexible "hinge" region. ATP binds in the cleft between these two lobes. The N-terminal lobe consists of a central β-sheet that houses the phosphate binding loop (called the "P-loop"), and a single α-helix, known as αC, the positioning of which is critical for kinase activity. The C-terminal lobe is mostly α-helical, and contains the catalytic loop containing the invariant Asn and Asp residues, and a metal binding loop containing an Asp required for coordination of the Mg^{2+} ion. RIO kinases contain an additional helix N-terminal (αR) to the canonical kinase N-lobe and a loop (the "flexible loop") inserted between the third β-strand of the N-lobe and αC. The purine nucleotide-binding site is also different from the canonical protein kinase binding pocket, particularly in the region that binds the γ-phosphate. RIO proteins contain subfamily-specific P-loops with sequences significantly different from those found in typical eukaryotic protein kinases. Crystal structures are available for the RIO kinases in an assortment of complexes, including ATP-Mg^{2+} bound, ADP-Mg^{2+}, and adenosine bound, as well as in the uncomplexed or "Apo" forms. Comparisons of these structures show a distinct difference in the positioning of the catalytic and metal binding loops between complexes in the presence and absence of the tri-phosphate or di-phosphate moiety. It should be also noted that there are also differences that specify RIO sub-family members. These features provide an additional opportunity to design not only general RIO-adapted but even RIO subtype-specific inhibitors.

It was shown that Rio1 and Rio2 kinases are subject to autophosphorylation on Ser residues [4,10,11]. The role of the autophosphorylation has not yet been established, though mutation of the autophosphorylation site of one Rio1 kinases did not affect the *in vitro* kinase activity [10]. Therefore, autophosphorylation may have a distinct function from that found in typical kinases — where the autophosphorylation regulates (usually increases or "switches on") the kinase activity. Rio1 can also phosphorylate myelin basic protein (MBP), casein and histone H1 in a non-specific fashion [10]. Up to date, little is known about possible natural substrates phosphorylated by RIO kinases.

The possible role of RIO kinases in pathogenesis, particularly in carcinogenesis, is not as well established as for many other kinases. However, the human Rio3 kinase (alternative name: *Homo sapiens* homologue to *Aspergillus nidulans* SUDD (suppressor of bimD6)) was found to be up-regulated in malignant melanomas [13], metastatic head and neck squamous cell carcinoma [14], as well as in pancreatic cancer [15], whereas human Rio1 kinase is up-regulated in colon cancer [16]. In addition, there is growing awareness that the efficiency of ribosome biogenesis plays an important role in rapidly proliferating cancer cells. A distinguishing characteristic of cancer cells is enlarged nucleoli, where rRNA synthesis and processing occur [17]. Several tumor suppressors (*e.g.* p53 and retinoblastoma) have been found to regulate the synthesis of new ribosomes [18]. There is a direct linkage between deregulation of ribosome biogenesis and malignant progression. For example, dysfunction of proteins engaged in ribosome biogenesis: ribosomal proteins (*e.g.* S19), nucleophosmin (NPM) and dyskeratosis congenita 1 protein (dyskerin1, DKC1) was strictly associated with many kinds of cancer [18]. Meaningfully, there is now a suggestion that Polymerase I (Pol 1), which is responsible for the transcription of rRNA, is a promising anti-cancer drug target. Pol 1 regulation by p53 links growth signals and ribosome biogenesis and its activity is specifically up-regulated in cancer cells, not just as a direct consequence of increased growth rates of cancer cells [19]. It was recently shown that inhibitors of rRNA synthesis exhibit significant anti-cancer activity. CX-3543, one such inhibitor, induces apoptosis in cancer cells and is under evaluation against carcinoid/neuroendocrine tumors in a phase II clinical trials [20]. CX-5461, another example, induces senescence and autophagy in solid tumor cell lines [20]. In summary, RIO proteins are emerging molecular targets in cancer that should be further validated and prospective targets for the future development of inhibitor-based anticancer therapies. In addition, the RIO kinase active site is sufficiently divergent from those of the large ePK family, which makes it likely that specific inhibitory molecules can be found [6]. Therefore, the purpose of this study was to identify preliminary scaffolds for the design of specific inhibitors of the Rio1 kinase that could serve as effective molecular probes. Due to the high level of conservation of the active site residues of the Rio1 kinases, we rationalized the Rio1 kinase from *Archaeoglobus fulgidus* (AfRio1) would be a suitable representative model for the entire Rio1 family (see Supplementary Fig. 1S for an alignment of the three RIO subfamilies).

A novel class of inhibitors of the JAK2/STAT3/STAT5 pathway was designed by Priebe et al. [21–23] using the scaffold of a natural product caffeic acid benzyl ester (CABE). This work led to the discovery of a series of potent inhibitors that include WP1066, WP1034, WP1130, WP1359, WP1130, and WP1193 targeting the p-STAT3 and p-STAT5. These inhibitors demonstrated the strong anti-tumor effect of these compounds in a wide range of *in vitro* and *in vivo* tumor models including melanoma [24–36]. Subsequent collaborative work led in part to the synthesis of compounds that are being tested here for their ability to bind and inhibit AfRio1 as a part of efforts to discover novel scaffolds for the design of RIO kinases inhibitors [37]. A small library of pyridine caffeic acid benzyl amide (CABA) derivatives was tested (Fig. 1), which allowed for identification of the functionalization that resulted in maximal inhibition of Rio1 autophosphorylation. Molecular docking simulations for these compounds and co-crystallization studies with a model compound WP1086, that contained all of the properties of the most active compounds, allowed identification of molecular interactions contributing to the effective inhibition. Interestingly, the structure demonstrated the model compound stabilizes a form of Rio1 previously observed bound to adenosine (ref) called the "inactive" form, which is distinct from the ATP-bound conformation, called the "active" form [38]. These results provide the basis for a rational approach to design of more specific, conformation-selective Rio1 inhibitors.

2. Materials and methods

2.1. Chemicals, reagents and synthesis of inhibitors

Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from BioShop. Radio-labeled adenosine-5′-triphosphate ([γ^{32}P]ATP) was from Hartmann Analytic. Ni-NTA agarose was obtained from Invitrogen. Benzonase nuclease solution was from Novagen. Commercially available inhibitor AG490, was purchased from Sigma-Aldrich. Unless otherwise stated all other chemicals were purchased from Sigma-Aldrich, Applichem or BioShop. Highly pure water, with resistivity of 18.2 MΩ·cm (Millipore) was used for all experiments.

2.1.1. Synthesis of selected inhibitors

^{1}H NMR spectra were recorded on Bruker Avanti 300 or Varian 300 MHz spectrometers in $CDCl_3$ and DMSO-d6 with TMS as the internal reference standard ^{13}C-NMR spectra were recorded on Varian 300 MHz, spectrometer DMSO-d6 with TMS as the internal reference standard MS spectra were determined using an Acuity UPLC-MS/MS (TQD-3) and AMD-604 mass spectrometers. Melting points were measured on Buchi melting point apparatus or Koefler apparatus and were uncorrected. Thin-layer chromatography (TLC) was carried on an aluminum sheet coated with SilicaGel 60F_{254} (EMD Chemicals Inc). Silica gel column chromatography was performed with CombiFlash