Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 308 (2003) 50–57
BBRC
www.elsevier.com/locate/ybbrc

Small molecule inhibitors of the RNA-dependent protein kinase

Narasimham V. Jammi,[1] Landon R. Whitby, and Peter A. Beal*

Department of Chemistry, University of Utah, Salt Lake City, UT 84112, USA

Received 24 June 2003

Abstract

The RNA-dependent protein kinase (PKR) is an interferon-induced serine/threonine protein kinase that phosphorylates the α subunit of the eukaryotic initiation factor 2 in response to viral infection. Classical genetic approaches for studying the role of PKR in cell signaling have their limitations due to overlapping but non-redundant pathways. Small molecule inhibitors of PKR will be useful in this regard. We report here, the discovery of a small molecule inhibitor of the kinase reaction of PKR. The inhibitor was discovered by screening a library of 26 different ATP-binding site directed inhibitors of varying structure. We also describe the development of a high-throughput assay for screening a large number of compounds for a PKR inhibitor using a rabbit reticulocyte lysate system and luciferase mRNA. The assay takes advantage of the fact that the reticulocyte lysate is rich in components of the translational machinery, of which PKR is an integral part. This assay can be carried out with added exogenous human PKR to study the effect of various compounds in their ability to rescue the translational block imposed by human PKR.

PKR is a serine/threonine protein kinase whose enzymatic activity is regulated through its interaction with RNA [1]. This protein has an approximately 20-kDa N-terminal RNA binding domain (RBD) and a C-terminal protein kinase domain. The RBD is composed of two copies of the dsRNA-binding motif (dsRBM I and dsRBM II), a sequence motif found in many dsRNA-binding proteins [2]. The functional properties of PKR are varied and PKR serves the organism in various ways. It has been suggested that PKR plays an important role in the normal maintenance of cell growth in the absence of any viral infection [3–5]. To test the importance of PKR in antiviral, antitumor, and apoptotic pathways at the whole organism level and to generate PKR-null cell lines for further investigation of PKR's role in cell signaling, two types of PKR-null mice have been generated [6,7]. In the PKR-null mice generated by Yang et al., the amino-terminal first two exons of the PKR gene were deleted. These mice were physically normal and the induction of type I IFN genes by poly[I:C] was not affected. However, the antiviral response induced by IFNγ and poly[I:C] was diminished. In the mice generated by Abraham et al., the carboxy-terminal region of the kinase domain of PKR was deleted. These mice are thus genetically ablated for functional PKR. These mice, similar to the ones generated by Yang et al., showed no apparent increase in their susceptibility to tumors. Antiviral response to both influenza and vaccinia viruses was also normal. In addition to this, the TNF-α-induced apoptosis pathway was not impaired. The authors explained these findings by invoking the presence of other redundant pathways, perhaps other eIF2-α kinases, which must have compensated for the loss of PKR function in these mice. These results underscore the need for additional approaches to modulate PKR activity in intact cells or whole animals for the purpose of studying PKR signaling. In addition to its obvious value in studying cell signaling, a small molecule inhibitor of PKR would also be useful to those studying RNA interference (RNAi), as the dsRNA-induced inhibition of translation can be blocked, thereby making it easier to observe RNAi in higher organisms.

* Corresponding author. Fax: 1-801-581-8433.
E-mail address: beal@chem.utah.edu (P.A. Beal).
[1] Present address: Department of Molecular Pharmacology, Stanford University, 318 Campus Drive, Clark Center, Stanford, CA 94305, USA.

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0006-291X(03)01318-4

2-Aminopurine (2-AP) is an inhibitor of PKR's kinase activity at millimolar concentrations and has been used to inhibit PKR in cells [8]. Unfortunately, 2-AP inhibits other kinases at these concentrations and the interpretation of cell-signaling data must take this into account [9]. To identify other small molecules capable of inhibiting PKR's kinase activity, 26 different ATP-binding site-directed inhibitors were tested for their ability to block RNA-induced PKR autophosphorylation. From the 26 small molecules that were tested, we found an inhibitor of PKR autophosphorylation that also rescues a PKR-induced translational block in a rabbit reticulocyte lysate system at micromolar concentrations. The small molecule that was able to inhibit the autophosphorylation reaction of PKR was an oxindole functionalized with an imidazole. In order to be able to screen for libraries with a large number of compounds for optimization of PKR affinity and selectivity, we also report a new high throughput screen for identifying small molecules that rescue the translation block imposed by human PKR.

Materials and methods

General. Distilled, deionized water was used for all aqueous reactions and dilutions. Biochemical reagents were obtained from Sigma/Aldrich unless otherwise noted. [γ-^{32}P]ATP (6000 Ci/mmol) was obtained from DuPont NEN. Imaging plates for storage phosphor autoradiography were purchased from Kodak. All data from phosphor imaging plates were obtained using a Molecular dynamics STORM 840 PhosphorImager and ImageQuant software. Liquid scintillation counting was carried out with a Beckman LS 6500 Scintillation Counter and Bio-Safe II cocktail from Research Products International.

Determination of IC_{50} for various inhibitors. Poly[I:C] at a concentration of 1 μg/mL was incubated with 68 ng PKR in 20 mM Tris–HCl (pH 7.6), 10 mM KCl, 10 mM $MgCl_2$, and 10% glycerol for 5 min at room temperature. [γ-^{32}P]ATP was added to a final concentration of 2 μM and the autophosphorylation reaction was allowed to proceed for 5 min at 30 °C in a final volume of 10 μL. To determine the IC_{50} of a compound, appropriate concentrations of the compound were added to the reaction mixture before addition of poly[I:C] and [γ-^{32}P]ATP. All reactions were quenched with an equal volume of 100 mM EDTA (pH 8.0), following which 10 μL of the quenched reaction was spotted onto phosphate cellulose filter paper discs (Whatman P81 filter paper, 2.5-cm diameter). The filter paper discs were then washed three times with 75 mM phosphoric acid to remove unincorporated ATP. The discs were air-dried for 30 min. The amount of radioactivity incorporated was determined by scintillation counting. Background activity was subtracted from each sample, following which the samples were normalized by subtracting the sample with the highest concentration of a compound from all other samples. These values were then reported as percent activity. The IC_{50} was determined by plotting percent activity versus the concentration, following which the data were fitted to the following equation: $\%\ \text{activity} = \{\text{range}/(1 + ([\text{compound}]/\text{IC}_{50})^{\text{slope}})\}$ using the least-squares method of Kaleidagraph. Each experiment was carried out in triplicate and the plotted values are average ± SD. To determine IC_{50} of an inhibitor using a standard gel assay, quenched autophosphorylation reactions in the presence of the inhibitor were electrophoresed on a 10% SDS–PAGE gel. The gel was then exposed to storage phosphor plates following which data were quantified as above.

In vitro translation of firefly luciferase in the presence of PKR/GST-PKR-KD and PKR inhibitors. Nuclease-treated rabbit reticulocyte lysate, luciferase mRNA, amino acid mixture, and Steady-Glow Luciferase substrate were purchased from Promega (Promega, WI). Approximately 68 ng of protein was aliquoted into a 96-well plate. Inhibitors were then added to the appropriate wells following which the mixture was allowed to incubate for 10 min at 30 °C. Where no inhibitor was required, a corresponding volume of water and DMSO (in the same ratio that each compound was dissolved in) was added to PKR. Rabbit reticulocyte lysate (8.5 μL) supplemented with 20 U of RNase inhibitor (RNasin; Promega, WI) was then added to each well and the reactions were further incubated for 10 min at 30 °C. Amino acids were then added to a final concentration of 1 mM. The translation reaction was initiated by addition of luciferase mRNA to a final concentration of 15 μg/mL. The reaction was allowed to proceed for 90 min at 30 °C, at the end of which 50 μL of Steady-Glow Luciferase substrate (Promega) was added to each well and mixed thoroughly by gently vortexing the 96-well plate. The reactions were then allowed to incubate at room temperature for 5 min before being counted in a luminometer (Wallac-Trilux 1450 Microbeta luminescence counter equipped with two signal detectors). The luminescence counts were then plotted as a histogram for each compound tested. Each histogram is an average of three experiments ± SD.

Expression and purification of PKR, yeast eIF2α, and GST-PKR-KD. The two forms of PKR (phospho- and dephospho-) and yeast eIF2α were expressed and purified as reported earlier [10]. *Escherichia coli* BL21 cells containing an expression vector for the GST-PKR-KD fusion protein were used to express the kinase domain. Primers with engineered restriction sites (*Bam*HI in the 5′ primer and *Xho*I for the 3′ primer) for the PKR kinase domain (amino acids 258–551) were synthesized chemically using an ABI automated DNA synthesizer. The primers were then incubated with a plasmid containing the full-length PKR gene and the region corresponding to amino acids 258–551 was amplified using PCR. The PCR product was then purified using standard phenol:chloroform extractions followed by ethanol precipitation. The coding region for the kinase domain was then digested with *Bam*HI and *Xho*I as also was a pGEX-5X-1 plasmid. The products of the restriction digest were purified on a 0.8% agarose gel, following which the plasmid and the coding region were ligated using T_4 DNA ligase. The ligation reactions were subsequently transformed into XL-1 blue cells, following which the DNA was harvested using a Qiagen MiniPrep spin column. The plasmid DNA thus obtained were then transformed into BL21 *E. coli* cells. BL21 *E. coli* cells containing the plasmid pGEX-5X-1-KD were then grown to an OD_{600} of 0.6 in LB containing 100 μg/mL ampicillin at 37 °C. Overexpression of the protein was induced using 0.3 mM IPTG and the cells were grown for an additional 4 h post-induction. The cells were then collected by centrifugation and resuspended using 20 mL lysis buffer (25 mM Tris, pH 8.0, 150 mM NaCl, 5% glycerol, 0.1% Triton X-100, 1 mM DTT, and 1 mM PMSF) per liter of cell culture. The cells were then subjected to snap freeze using liquid nitrogen. The cells were stored overnight at −80 °C and then allowed to thaw at room temperature for 1 h. The cell contents were released using a French press at a psi of 10,000–15,000. This process was repeated three times to ensure maximum lysis. The cell lysate was clarified by centrifugation for 30 min at 27,000*g*. Lysate was then incubated with pre-washed glutathione–Sepharose 4B beads (Pharmacia) at a stoichiometry of 1.2 mL beads per liter of cell culture for 2 h at 4 °C. Following incubation, the samples were gently centrifuged and the supernatant discarded. Samples were then washed four times with 7 mL high salt lysis buffer (lysis buffer supplemented with 350 mM NaCl) per 600 μL glutathione–Sepharose beads. The beads were then loaded onto Poly-Prep chromatography columns (Bio-Rad, 0.8 × 4.0 cm) at a volume of

600 μL per column. GST-PKR-KD was then eluted from the beads using 2 mL elution buffer (25 mM Tris, pH 8.0, 500 mM NaCl, 100 mM reduced glutathione, 5% glycerol, 0.1% Triton X-100, 10 mM DTT, and 1 mM PMSF). Samples were dialyzed overnight into storage buffer (25 mM Tris, pH 8.0, 350 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM DTT, and 1 mM PMSF) using Slide-A-Lyser dialysis cassettes (Pierce) (MWCO 10,000). Protein samples were quantified by electrophoresis of samples and known amounts of bovine serum albumin (BSA) on 10% SDS–PAGE gel. Samples were visualized by SyproOrange (Bio-Rad) staining and a Molecular Dynamics STORM 840 PhosphorImager and bands were quantified using ImageQuant software. A standard curve was generated from the BSA bands and this standard curve was then used to determine the concentration of GST-PKR-KD.

Determination of the specific activity of GST-PKR-KD. Specific activity of GST-PKR-KD was determined using the protocol described in an earlier paper [10]. Briefly, 68 ng GST-PKR-KD in 20 mM Tris–HCl (pH 7.6), 10 mM KCl, 10 mM $MgCl_2$, and 10% glycerol was incubated with 2 μM [γ-^{32}P]ATP for 5 min at 30 °C, following which eIF2α was added to a final concentration of 500 nM. Aliquots of the reaction mixture were removed at 30, 60, 90, and 120 s and quenched with pre-heated (95 °C) SDS–PAGE gel loading buffer. The protein samples were electrophoresed on a 13.5% SDS–PAGE gel and the labeled proteins were visualized and band intensities quantified using a STORM PhosphorImager and ImageQuant software. To determine the molar amount of phosphate incorporated into eIF2α per unit time, bands were cut from the SDS–PAGE gels and scintillation counted. A standard curve correlating counts per minute to moles of phosphate was obtained using serial dilutions of the [γ-^{32}P]ATP used in each reaction. Rates were obtained from the slopes of linear fits to the data plotted as nanomolar phosphate incorporated into eIF2α per minute. Each experiment was carried out in triplicate and plotted values are averages of three experiments.

Results

GST-PKR-KD was expressed and purified and was found to be active

To facilitate the study of PKR's in vitro kinase activity, we overexpressed the kinase domain (amino acids 258–551) of human PKR fused C-terminal to glutathione-*S*-transferase. These amino acids were chosen because they have maximum homology to the well-characterized kinase domain of PKA. Also, Dever and co-workers [11] have demonstrated that a fusion protein consisting of amino acids 258–551 and GST was active in yeast. GST-kinase domain (GST-PKR-KD) was expressed and purified to near homogeneity (Fig. 1A). We next investigated the eIF2-α-kinase activity of this protein. Not surprisingly, the fusion protein was an active kinase in terms of eIF2α phosphorylation (Fig. 1B (top)). Specific activity was determined using an in vitro phosphorylation assay (Fig. 1B (bottom)). The specific activity was determined to be 1.4 ± 0.3 nM/min/68 ng GST-PKR-KD. This is ~8 times lower than the constitutively active full-length phosphoPKR isolated from a similar bacterial overexpression system [10].

Small molecules that inhibit PKR autophosphorylation were identified from a small library

The activity of protein kinases has been measured by a variety of techniques. A widely used procedure to achieve separation of unreacted ATP from the phosphorylated kinase substrate is by precipitating phosphoprotein onto cellulose strips by trichloroacetic acid followed by extensive washing [12]. Roskoski [13] developed an alternative procedure for resolving phosphohistone from ATP and its metabolites based upon the adsorption of phosphohistone onto phosphocellulose strips. We decided to adapt this procedure to our kinase assays in which instead of phosphohistone we would selectively adsorb phosphoPKR. Activatable dephosphoPKR was obtained using a previously reported protocol [10]. In order to activate dephosphoPKR, the protein was pre-incubated with poly[I:C] at a concentration of 1 μg/mL,

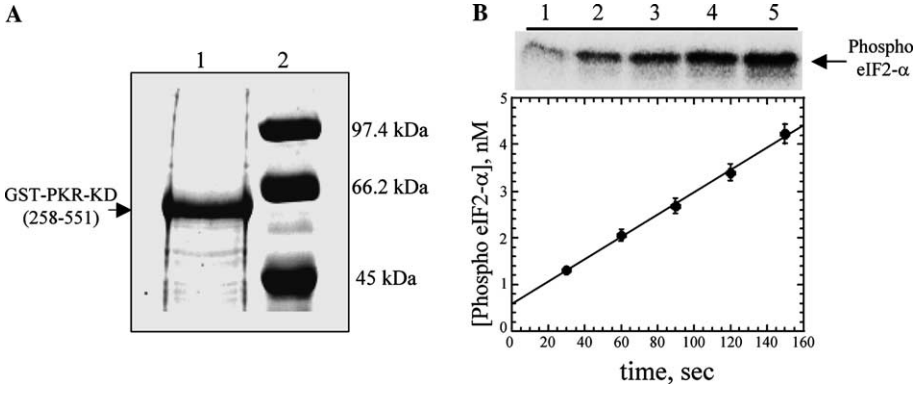

Fig. 1. (A) SyproOrange stained gel used to resolve the kinase domain of PKR as a GST fusion protein (GST-PKR-KD) after purification. Lane 1, GST-tagged kinase domain of PKR (amino acids 258–551) overexpressed in *E. coli* and lane 2, protein molecular weight markers. (B) Time course for the phosphorylation of eIF2-α by GST-PKR-KD. (Top) Storage phosphor autoradiograms of SDS–PAGE gels used to resolve products from kinase reactions with the kinase domain of PKR. Lanes 1–5: lane 1, 30-s time point; lane 2, 60-s time point; lane 3, 90-s time point; and lane 4, 120-s time point. (Bottom) Plot of product formation as a function of time. The data were fit to a line using KaleidaGraph. Data points reported are the average for three independent experiments. The specific activity of the GST-PKR-KD sample was determined to be 1.4 ± 0.3 nM/min/68 ng GST-PKR-KD.

following which autophosphorylation was allowed to proceed in the presence of [γ-^{32}P]ATP. Twenty-six different ATP-binding site directed inhibitors (Glaxo-SmithKline) were tested for their ability to block this RNA-induced PKR autophosphorylation. A broad range of values was observed with the most potent compounds inhibiting with IC_{50}s near 100 nM and no inhibition observed with other compounds at 100 μM (Table 1). For example, compounds 2 and 20 were the best inhibitors with IC_{50}s of 100 ± 10 and 120 ± 10 nM, respectively, while compounds 3, 11, 12, 13, 14, 22, and 26 did not inhibit the autophosphorylation reaction even at 100 μM (Table 1). All other compounds had IC_{50}s whose values were between 100 nM and 100 μM. For further study, we chose the structurally simplest high affinity inhibitor (compound #16; $IC_{50} = 210 \pm 10$ nM) from the library, repeated the experiment, and analyzed the products on a 10% SDS–PAGE gel. The IC_{50} estimated for compound 16 from the gel assay was 186 ± 20 nM (see Fig. 2 for comparison).

A novel high throughput screen was developed that facilitates the screening of PKR inhibitors

The potency of inhibitors of PKR's kinase activity can be assayed by directly measuring their effect on autophosphorylation of the enzyme as described above. However, these experiments require radioisotopes and are difficult to adapt for high throughput analysis of a large number of compounds at different concentrations. It has been known for more than a decade that PKR activity blocks translation in rabbit reticulocyte lysates and that PKR inhibitors are able to rescue this block [14]. Thus, PKR activity could be measured in reticulocyte lysates photometrically by measuring the activity of firefly luciferase in an in vitro translation reaction with added luciferase mRNA. We tested the efficacy of this approach by first demonstrating that EBER1 RNA, a potent virally derived RNA inhibitor of PKR, rescues inhibition of luciferase translation induced by adding dsRNA (poly[I:C]) to rabbit reticulocyte lysate, con-

Table 1
Table of IC_{50} values for inhibition of PKR autophosphorylation[a]

Compound #	IC_{50} (μM)	Compound #	IC_{50} (μM)	Compound #	IC_{50} (μM)
2	0.10 ± 0.01	9	0.90 ± 0.17	4	12.5 ± 6.8
20	0.12 ± 0.01	24	1.5 ± 0.5	3	>100
7	0.16 ± 0.07	8	1.7 ± 0.5	11	>100
6	0.18 ± 0.02	23	2.0 ± 0.5	12	>100
16	0.21 ± 0.04	18	3.3 ± 1.4	13	>100
25	0.22 ± 0.07	17	3.7 ± 1.0	14	>100
19	0.35 ± 0.10	21	4.0 ± 1.0	22	>100
10	0.35 ± 0.02	5	4.3 ± 2.3	26	>100
15	0.73 ± 0.06	1	4.4 ± 2.8		

[a] Structures for the above compounds may be obtained as Supplementary Information via email from the corresponding author.

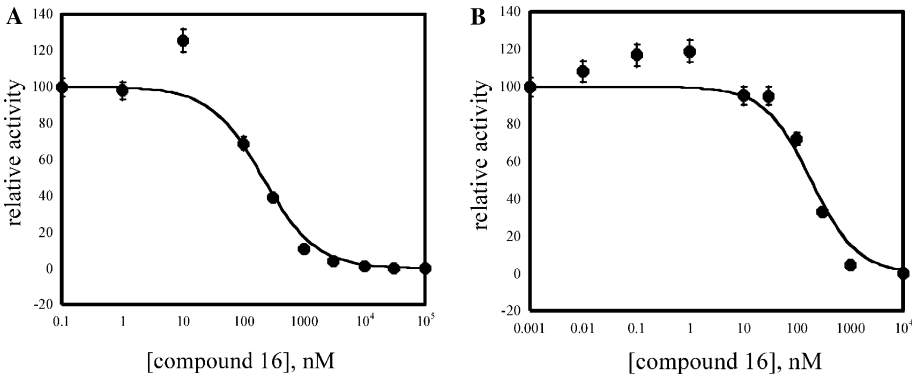

Fig. 2. Plot of relative activity of dephosphorylated PKR as a function of concentration of compound #16. Each reaction had 68 ng dephosphorylated PKR, 1 μg/mL activating RNA (poly[I:C]), and 2 μM [γ-^{32}P]ATP. The data were fit to the equation: % activity = $\{\text{range}/(1 + ([\text{TFO}]/IC_{50})^{\text{slope}})\}$ using KaleidaGraph. Data points reported are the average ± SD for three independent experiments. (A) An IC_{50} curve for the inhibition of the autophosphorylation reaction of PKR by compound 16 using a P81 phosphocellulose paper assay (for a detailed description, see Materials and methods). (B) An IC_{50} curve for the inhibition of the autophosphorylation reaction of PKR by compound #16 as determined by a gel assay. The IC_{50}s as determined by both methods were similar (compare 210 ± 10 nM by the paper assay with 186 ± 20 nM by the gel assay).

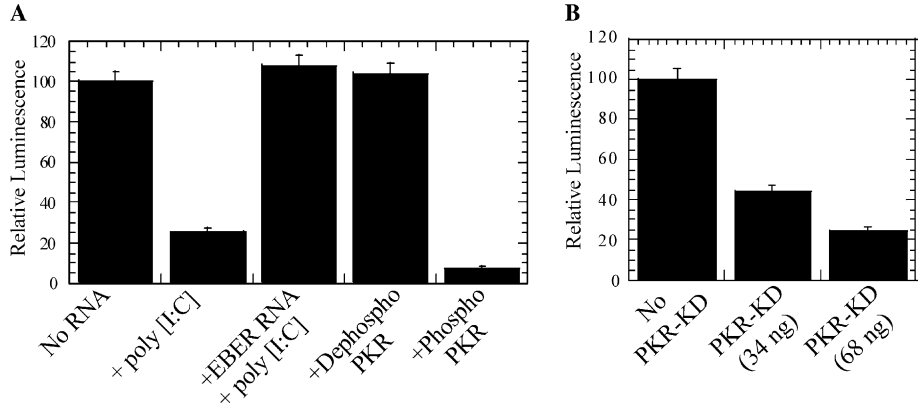

Fig. 3. (A) Measurement of PKR activity using a luminescence assay. Activity of PKR is measured photometrically by monitoring the luciferase activity arising due to the translation of luciferase mRNA. Each reaction contains 8.5 μL of rabbit reticulocyte lysate, 15 μg/mL of luciferase mRNA, 1 mM amino acids, and the indicated compound as described below. No RNA, no activating RNA (poly[I:C]) added. The translation reaction is allowed to proceed under conditions where there is no inhibition of endogenous rabbit PKR; +poly[I:C], an activating RNA, poly[I:C] at a concentration of 1 μg/mL, was added to activate endogenous rabbit PKR; +EBER RNA + poly[I:C], EBER RNA (10 μM) was added to inhibit the activity of rabbit PKR; +dephosphoPKR, dephosphorylated PKR (34 ng) obtained via overexpression in bacterial cells [10] was added in the absence of an activating RNA; and +phospho PKR, phosphorylated PKR (34 ng) [10] was added to the reaction. (B) Inhibition of translation by addition of the kinase domain of PKR fused to GST (GST-PKR-KD). Each reaction contains 8.5 μL of rabbit reticulocyte lysate, 15 μg/mL of luciferase mRNA, 1 mM amino acids, and the indicated compound as described below. No GST-PKR-KD, no exogenous protein is added; 34 ng GST-PKR-KD, and 68 ng GST-PKR-KD, GST-PKR-KD was added to the reaction at the indicated amounts.

firming that the effect is PKR-dependent (Fig. 3A) [14,15]. Also, we determined the effect of adding purified human PKR overexpressed in *E. coli* without additional dsRNA. As expected, if the enzyme is dephosphorylated prior to addition to the lysate, no inhibition of translation is observed (Fig. 3A). However, the addition of phosphoPKR, in the absence of dsRNA, leads to potent inhibition of translation (Fig. 3A). Next, GST-PKR-KD was used to see if the exogenously added kinase domain by itself could inhibit translation. Indeed, increasing concentrations of GST-PKR-KD led to an incremental inhibition of translation (Fig. 3B). Once it was established that GST-PKR-KD could inhibit the in vitro translation reaction, we chose to use GST-PKR-KD instead of full-length PKR, since it was easier to purify GST-PKR-KD and we were able to obtain GST-PKR-KD in larger amounts than full-length PKR. We used four inhibitors (2-aminopurine, staurosporine, compound #16, and compound #22) to evaluate their potency in inhibiting the kinase domain, as evidenced by a rescue of translation. Each compound was tested over a wide range of concentrations (Fig. 4). 2-Aminopurine, a known PKR inhibitor with an IC_{50} of 10 mM, rescued translation to ~70% at a concentration of 10 mM (Fig. 4A). Staurosporine (a PKC inhibitor) displayed an overall decrease in translation with increasing concentrations (Fig. 4C). Compound #16, which is one of the better inhibitors from the library screen ($IC_{50} = 210 \pm 10$ nM), was able to rescue translation to 50% of the control reaction at a concentration of 10 μM. There was no further appreciable rescue at higher concentrations (Fig. 4B). Importantly, half maximal rescue of translation was obtained at a concentration of near 100 nM. Compound #22, a compound that failed to show any inhibition of the autophosphorylation reaction of PKR, did not rescue translation (Fig. 4D).

Discussion

Initial interest in protein kinases as pharmacological targets was stimulated by the findings that many viral oncogenes encode structurally modified cellular protein kinases with constitutive enzyme activity, leading to a number of important human diseases including cancer and disorders of the human immune system [16,17]. PKR is a kinase that has an important role in not only interferon-mediated viral immunity, but also in apoptosis and cell growth [3,18]. Expression of a catalytically inactive mutant of PKR in NIH-3T3 cells leads to their malignant transformation and formation of tumors in nude mice and promotes growth in HeLa cells [3,4,19]. Additional evidence supporting a role for PKR in the control of cell growth and differentiation has arisen from stable high-level expression of wild-type and variant, non-functional forms of PKR in yeast and cultured mammalian cells [20]. Stable expression of wild-type PKR suppresses growth in yeast cells, accompanied by increased eIF2-α phosphorylation [21,22]. The expression of mutant forms of PKR results in the down-regulation of the endogenous wild-type enzyme in NIH-3T3 cells.

Interestingly, mice that were genetically ablated for functional PKR showed no apparent increase in their

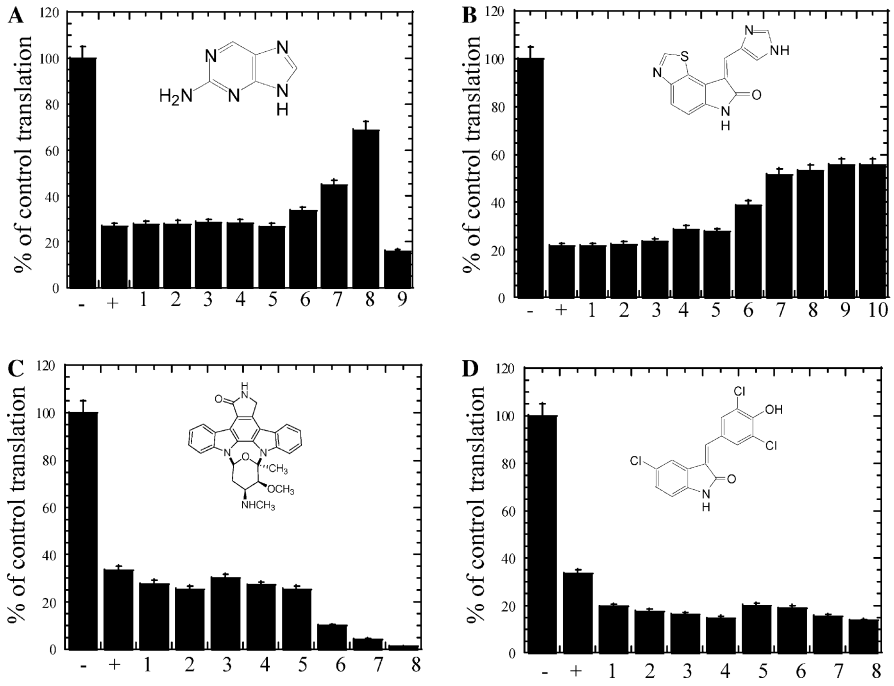

Fig. 4. Effect of ATP-site directed inhibitors on the GST-PKR-KD imposed translation block. All reactions contain 8.5 μL of rabbit reticulocyte lysate, 15 μg/mL of luciferase mRNA, 1 mM amino acids, and the indicated compound as described below. (−), no exogenous protein added; (+), 34 ng GST-PKR-KD was added to the reaction (A) Rescue of the translation block imposed by GST-PKR-KD by 2-aminopurine (2-AP). Lanes 1–9, 2-aminopurine was added to a final concentration of 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM, 1 mM, 10 mM, and 100 mM, respectively. (B) Rescue of the translation block imposed by GST-PKR-KD by compound #16. Lanes 1–10, compound #16 was added to a final concentration of 0.001 nM, 0.01 nM, 0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 50 μM, and 100 μM, respectively. (C) Effect of the commercially available PKC inhibitor, staurosporine, on the translation block imposed by GST-PKR-KD. Lanes 1–8, staurosporine was added to a final concentration of 0.001 nM, 0.01 nM, 0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM, respectively. (D) Effect of compound #22 on the GST-PKR-KD imposed translation block. Lanes 1–8, compound #22 was added to a final concentration of 0.001 nM, 0.01 nM, 0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM, respectively.

susceptibility to tumors [6,7]. This could be due to redundant eIF2-α kinases compensating for the role of PKR in these mice. There is therefore, a need to modulate PKR activity exclusively in order to study PKR signaling. As an initial approach to address this problem, we tested a battery of 26 different small molecule inhibitors obtained through a collaboration with GlaxoSmithKline. We employed a phosphocellulose paper assay to determine the IC_{50}s of these compounds for inhibition of PKR's kinase activity. This is a standard method used to measure kinase activity [13]. A broad range of IC_{50} values was observed with the most potent compounds inhibiting with IC_{50}s near 100 nM and no inhibition observed with other compounds (see Table 1).

The above-mentioned phosphocellulose paper assay may not prove to be practical when a high throughput screen is required for multiple candidate inhibitors at different concentrations. We therefore developed an in vitro reticulocyte lysate system wherein we could measure the effect of exogenously added inhibitors as evidenced by a rescue of the translation block due to the activity of PKR. Our initial challenge lay in establishing a system that reproducibly demonstrated a translation block due to the catalytic activity of PKR followed by its corresponding rescue by a known PKR inhibitor. The effect of other PKR inhibitors could then be assessed by measuring their ability to rescue this PKR-imposed translation block in luciferase translation and comparing it to the known PKR inhibitor. The PKR inhibitor that we used was 2-aminopurine. As mentioned earlier, 2-aminopurine is an inhibitor of PKR with an IC_{50} of approximately 10 mM [23]. Indeed, upon addition of increasing concentrations of 2-aminopurine, we saw a gradual rescue of the translation block induced by the kinase domain of PKR, with maximal rescue at 10 mM (Fig. 4A). At 100 mM 2-aminopurine, there was an overall decrease in translation. We attribute the decrease in translation in our assay at 100 mM 2-aminopurine to the nonspecific inhibition of other components in the translation machinery at these high concentrations. As mentioned earlier, we have tested a battery of compounds for their inhibition of the autophosphorylation reaction of PKR. Some of these compounds were able to inhibit the autophosphorylation reaction of PKR with IC_{50}s in the 100–300 nM range. To demonstrate the efficacy of the in vitro translation assay, we decided to test compound #16. Not surprisingly, we saw a gradual

rescue of the translation block imposed by GST-PKR-KD with maximal rescue at 10 μM of compound #16 (Fig. 4B). As a control, we performed the same experiment with compound #22, a compound that did not inhibit PKR's autophosphorylation reaction. Indeed, compound #22 did not rescue the translation block imposed by GST-PKR-KD. Another commercially available kinase inhibitor, staurosporine was tested for its ability to rescue translation. Staurosporine, a known PKC inhibitor, and an ATP-site binding alkaloid, exhibits poor selectivity despite its high potency [24]. As can be seen in Fig. 4C, staurosporine does not rescue translation. In fact, at higher concentrations, staurosporine leads to an overall decrease in translation, presumably due to non-specific inhibition of other components of the translation machinery.

These results have several important implications for our future work. First, they indicate that we can supplement this rabbit reticulocyte lysate system with active GST-PKR-KD and potentially identify inhibitors of the kinase activity of human PKR. These results also provide insights into developing new inhibitors, in terms of the scaffold that could be used. For instance, 2-aminopurine was the compound that displayed maximal rescue of translation in the in vitro assay. However, it was only able to rescue translation at relatively high concentrations (10 mM). On the other hand, compound #16 was able to rescue translation to about 50% at a concentration of 10 μM. Thus, this relatively simple compound can effectively inhibit PKR at micromolar concentrations with a selectivity sufficient to allow translation to occur in the reticulocyte lysate system. This stands in contrast to staurosporine, which inhibits PKR activity under these conditions in vitro ($IC_{50} = 17$ nM, data not shown), but blocks translation in these lysates at PKR inhibitory concentrations (Fig. 4C). Thus, the oxindole/imidazole functionality found in compound #16 provides a useful core scaffold for the development of chemical libraries to be screened for more potent/selective PKR inhibitors. Such libraries will be effectively screened for PKR inhibitors using the luciferase translation assay described here.

Acknowledgments

The authors thank GlaxoSmithKline for the library of compounds used in this study. The authors also acknowledge a grant from the National Institutes of Health (GM57214) for funding this research.

References

[1] J. Galabru, A. Hovanessian, Two interferon-induced proteins are involved in the protein kinase complex dependent on double-stranded RNA, Cell 43 (1985) 685–694.

[2] I. Fierro-Monti, M.B. Mathews, Proteins binding to duplexed RNA: one motif, multiple functions, Trends Biochem. Sci. 25 (2000) 241–246.

[3] A.E. Koromilas, S. Roy, G.N. Barber, M.G. Katze, N. Sonenberg, Malignant transformation by a mutant of the interferon-inducible double-stranded RNA dependent protein-kinase, Science 257 (1992) 1685–1689.

[4] E.F. Meurs, J. Galabru, G.N. Barber, M.G. Katze, A.G. Hovanessian, Tumor suppressor function of the interferon-induced double-stranded RNA-activated protein kinase, Proc. Natl. Acad. Sci. USA 90 (1993) 232–236.

[5] S. Davis, J.C. Watson, In vitro activation of the interferon-induced, double-stranded RNA-dependent protein kinase PKR by RNA from the 3′ untranslated regions of human α-tropomysin, Proc. Natl. Acad. Sci. USA 93 (1996) 508–513.

[6] Y.-L. Yang, L.F.L. Reis, J. Pavlovic, A. Aguzzi, R. Schafer, A. Kumar, B.R.G. Williams, M. Ague, C. Weissmann, Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase, PKR, EMBO J. 14 (1995) 6095–6106.

[7] N. Abraham, D.F. Stojdl, P.I. Duncan, N. Methot, T. Ishii, M. Dube, B.C. Vanderhyden, H.L. Atkins, D.A. Gray, M.W. McBurney, A.E. Koromilas, E.G. Brown, N. Sonenberg, J.C. Bell, Characterization of transgenic mice with targeted disruption of the catalytic domain of the double-stranded RNA-dependent protein kinase, PKR, J. Biol. Chem. 274 (1999) 5953–5962.

[8] J. Huang, R.J. Schneider, Adenovirus inhibition of cellular protein synthesis is prevented by the drug 2-aminopurine, Proc. Natl. Acad. Sci. USA 87 (1990) 7115–7119.

[9] K. Posti, S. Leinonen, S. Tetri, S. Kottari, P. Viitala, O. Pelkonen, H. Raunio, Modulation of murine phenobarbital-inducible CYP2A5, CYP2B10 and CYP1A enzymes by inhibitors of protein kinases and phosphatases, Eur. J. Biochem. 264 (1999) 19–26.

[10] N.V. Jammi, P.A. Beal, Phosphorylation of the RNA-dependent protein kinase regulates its RNA-binding activity, Nucleic Acids Res. 29 (2001) 3020–3029.

[11] T.L. Ung, C. Cao, J. Lu, K. Ozato, T.E. Dever, Heterologous dimerization domains functionally substitute for the double-stranded RNA binding domains of the kinase PKR, EMBO J. 20 (2001) 3728–3737.

[12] J.D. Corbin, E.M. Reimann, Assays of protein kinase, Methods Enzymol. 38 (1983) 287–293.

[13] R. Roskoski Jr., Assays of protein kinase, Methods Enzymol. 38 (1983) 3–6.

[14] P.A. Clarke, N.A. Sharp, M.J. Clemens, Translation control by the Epstein–Barr virus small RNA EBER-1: reversal of the double-stranded RNA-induced inhibition of protein synthesis in reticulocyte lystates, Eur. J. Biochem. 193 (1990) 635–641.

[15] T.V. Sharp, M. Schwemmle, I. Jeffrey, K. Laing, H. Mellor, Comparative analysis of the regulation of the interferon-inducible protein kinase PKR by Epstein–Barr virus RNAs EBER-1 and EBER-2 and adenovirus VAI RNA, Nucleic Acids Res. 21 (1993) 4483–4490.

[16] K.S. Kolibaba, B.J. Druker, Protein tyrosine kinases and cancer, Biochim. Biophys. Acta 1333 (1997) 217–248.

[17] R.M. Perlmutter, S.D. Levin, M.W. Appleby, S.J. Anderson, J. Alberola-Ila, Regulation of lymphocytes by protein phosphorylation, Ann. Rev. Immunol. 11 (1993) 451–499.

[18] S.B. Lee, M. Esteban, The interferon-induced double-stranded RNA-activated protein kinase induces apoptosis, Virology 199 (1994) 491–496.

[19] G.N. Barber, M. Wambach, S. Thompson, R. Jagus, M.G. Katze, Mutants of the RNA-dependent protein kinase (PKR) lacking double-stranded RNA binding domain I can act as transdominant inhibitors and induce malignant transformation, Mol. Cell. Biol. 15 (1995) 3138–3146.

[20] R. Jagus, B. Joshi, G.N. Barber, PKR, apoptosis and cancer, Int. J. Biochem. Cell Biol. 31 (1999) 123–138.

[21] K.L. Chong, L. Feng, K. Schappert, E. Meurs, T.F. Donahue, J.D. Friesen, A.G. Hovanessian, B.R.G. Williams, Human p68 kinase exhibits growth suppression in yeast and homology to the translational regulator GCN2, EMBO J. 11 (1992) 1553–1562.

[22] T. Dever, J.-J. Chen, G.N. Barber, A.M. Cigan, L. Feng, T.F. Donohue, I.M. London, M.G. Katze, A.G. Hinnebusch, Mammalian eIf-2a-PKs functionally substitute for GCN2 and stimulate GCN4 translation in yeast, Proc. Natl. Acad. Sci. USA 90 (1993) 4616–4620.

[23] Y. Hu, T.W. Conway, 2-Aminopurine inhibits the double-stranded RNA-dependent protein kinase both in vitro and in vivo, J. Interferon Res. 13 (1993) 323–328.

[24] S. Omura, Y. Sasaki, Y. Iwai, H. Takeshima, Staurosporine, a potentially important gift from a microorganism, J. Antibiot. (Tokyo) 48 (1995) 535–548.