Selection of Small-Molecule Mediators of the RNA Regulation of PKR, the RNA-Dependent Protein Kinase

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The RNA-dependent protein kinase (PKR) is a component of the interferon antiviral response and a member of the class of RNA-binding proteins with a double-stranded RNA binding motif. PKR is activated when it binds to double-stranded RNA (dsRNA) or viral replicative intermediates that comprise dsRNA and this activation results in the inhibition of protein synthesis. Some viruses circumvent this activity through the synthesis of highly structured decoy RNAs that bind PKR and block activation. Small-molecule mediators of the binding of PKR to these RNA inhibitors would be useful tools to further define the importance of specific PKR–RNA complexes in vivo and may possess antiviral activity. Here we investigate the ability of a library of structurally diverse peptide–acridine conjugates (PACs) to target a complex formed between the dsRNA binding domain (dsRBD) of PKR and a viral RNA inhibitor. We used a novel screening method based on the cleavage of RNA ligands with ethylenediaminetetraacetic acid·Fe modified protein. The selection revealed a PAC (9-anilinoacridine-4-Hyp-Nap-Nap, where Hyp is trans-4-hydroxyproline and Nap is 1-napthylalanine), able to inhibit the binding of the PKR dsRBD to RNA with an IC_{50} value of $10 \pm 5\ \mu M$. Furthermore, the structural requirements for inhibition by the selected PAC were substantiated in an independent PKR activation assay. We found that the potency of inhibition by an intercalating ligand can be increased by the introduction of a substituent that does not increase the overall charge of the molecule. This result is important for the design of inhibitors of PKR–RNA binding that function inside living cells.

KEYWORDS:

antiviral agents · bioorganic chemistry · combinatorial chemistry · peptides · RNA recognition

Introduction

The RNA-dependent protein kinase (PKR) is an interferon-inducible enzyme that is involved in protein synthesis inhibition and the antiviral response in human cells.[1–4] This serine/threonine kinase is activated during viral infection by binding to double-stranded RNA (dsRNA) through a mechanism that involves autophosphorylation.[5–8] Once activated, PKR can subsequently phosphorylate the alpha subunit of the translation initiation factor eIF2 and repress protein synthesis.[9–11] Viruses have evolved a variety of different mechanisms to inactivate this kinase. As a counter measure to PKR, adenovirus and Epstein Barr virus synthesize RNAs (VA and EBER, respectively) with extensive duplex structure that competitively bind PKR and block activation.[12–14]

Regulation of the activity of PKR by kinase activating and inhibiting RNAs involves a sequence motif known as the dsRNA binding motif (dsRBM) found in many dsRNA-binding proteins.[15] PKR ($\sim$68 kDa) contains 2 copies of this dsRBM located in the N-terminal dsRNA binding domain (dsRBD) of the protein.[16–17] Enzymes that contain this type of binding module are involved in a multitude of biological processes that range from RNA editing to translational regulation.[18–23]

RNA-binding molecules that inhibit the formation of protein–RNA complexes have the potential to serve both as research tools and as therapeutic agents that interfere with the function of specific RNAs.[24–31] For instance, compounds with the ability to target and disrupt the complexes formed between specific viral inhibitory RNAs and PKR would aid in the study of the role these RNAs play at various points in the process of viral infection. In addition, these compounds would have the potential to be developed into new antiviral agents. For this purpose, an inhibitor should be cell permeable, able to bind the target RNA with high affinity, and should prevent the association of the RNA-binding protein. However, the number of molecules able to inhibit of the formation of specific protein–RNA complexes is rather limited.[32–34] We showed previously that triple-helix-forming oligonucleotides can be used to prevent the interaction between PKR and regulatory RNA ligands.[35] However, the charge, molecular weight, and stability of these oligoribonucleotides make it unlikely that they could function in vivo. Therefore, we are developing a different class of RNA-binding molecules which may have the desired properties.

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The 9-anilinoacridines comprise a class of small molecules that are known to intercalate into DNA and inhibit topoisomerase II.[36] Analogues such as the antitumor drug amsacrine are able to permeate into the cell and are highly potent against P388 leukemia and Lewis lung carcinoma.[37–38] Recently, 9-anilinoacridines have also been shown to bind duplex RNA by intercalation.[39] Our approach to the development of high-affinity RNA-binding compounds is to synthesize an acridine intercalator that is modified with peptide appendages such that the peptide functional groups are directed into the grooves of the RNA structure in the bound complex. In this way, additional affinity could arise from the introduction of stabilizing interactions between the peptide and the unique surface of the RNA duplex groove. Furthermore, the peptide appendage could make stabilizing contacts at bulged, mismatched, or looped nucleotides found adjacent to the intercalation site in more structurally complex RNA targets. We previously reported the preparation of two acridine-based monomers capable of directing structurally diverse peptide substituents into nucleic acid grooves.[40–41] Both compounds are compatible with standard solid-phase peptide synthesis (SPPS) procedures and can be readily integrated into the synthesis of combinatorial libraries of peptide – acridine conjugates (PACs).

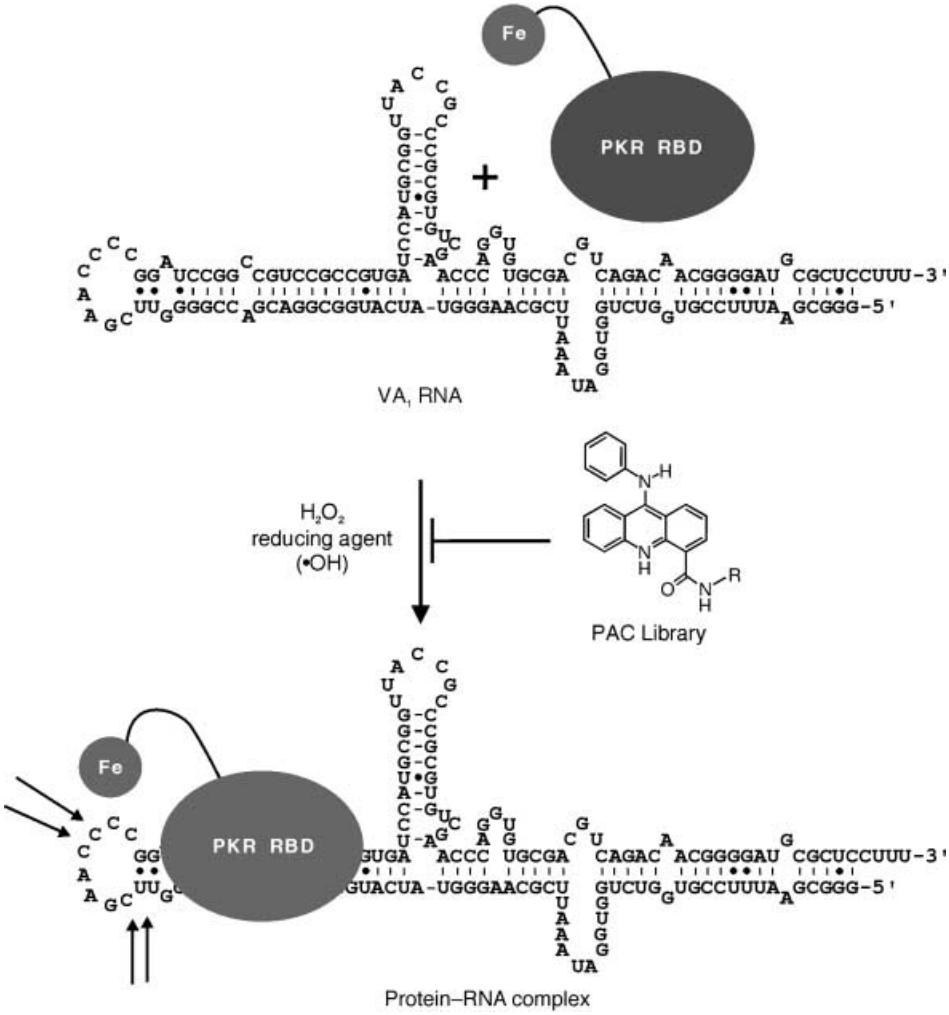


Figure 1. *Schematic diagram of the protein affinity cleavage screening assay for inhibitors of formation of a protein – RNA complex. The RNA-binding domain of PKR is modified with the hydroxyl radical generator EDTA·Fe and, when allowed to bind VA$_I$ RNA under reaction conditions that produce hydroxyl radicals, the protein cleaves the RNA in specific regions. Acridine libraries can be introduced along with the protein and RNA to test for inhibition of the affinity cleavage reaction, which signifies disruption of the protein – RNA complex.*

In order to screen libraries of these compounds for their ability to disrupt the formation of a PKR – RNA complex, we employed an assay developed to monitor the RNA-binding properties of the dsRBD of PKR (Figure 1). Previously, we site-specifically modified the PKR dsRBD with ethylenediaminetetraacetic acid (EDTA)·Fe and applied the technique of affinity cleavage to characterize structural features of the complex formed between PKR and VA$_I$ RNA, an inhibitor of the PKR kinase activity generated by adenovirus.[42] This binding assay was here used to screen a 108-compound library of PACs. We find that the potency of inhibition of the PKR-dsRBD – VA$_I$-RNA complex by these PACs varies depending on the structure of the peptide. Additionally, relative potencies are observed in a separate PKR activation assay. These studies indicate that the efficacy of inhibition by an intercalative ligand can be increased without increasing the overall charge of the molecule. This result is important for the design of inhibitors of PKR – RNA binding that can permeate into cells.

Results and Discussion

Preliminary screening

DNase I footprinting has been used to select small molecules from mixtures for their ability to target specific DNA sequences.[43] In order to assess whether our affinity cleavage assay could similarly determine the relative potencies of inhibition, we first tested four different small molecules for their ability to prevent the PKR dsRBD from binding adenovirus VA$_I$ RNA. The classical intercalator 9-aminoacridine (9-AA, Figure 2a) is commercially available and has been shown by X-ray crystallography to intercalate into dsRNA.[44] To evaluate the importance of the aniline substitution at the 9-position and of a simple carboxamide at the 4-position, 9-anilinoacridine-4-(N-methyl)carboxamide (9-Ani, Figure 2a) was prepared in our laboratory. Ser-Val-Acr-Lys (SVAcrK, Figure 2a) is a PAC that we previously synthesized and whose binding to duplex nucleic acids was demonstrated.[36] Ser-Val-Lys-Lys (SVKK, Figure 2a) was designed to test

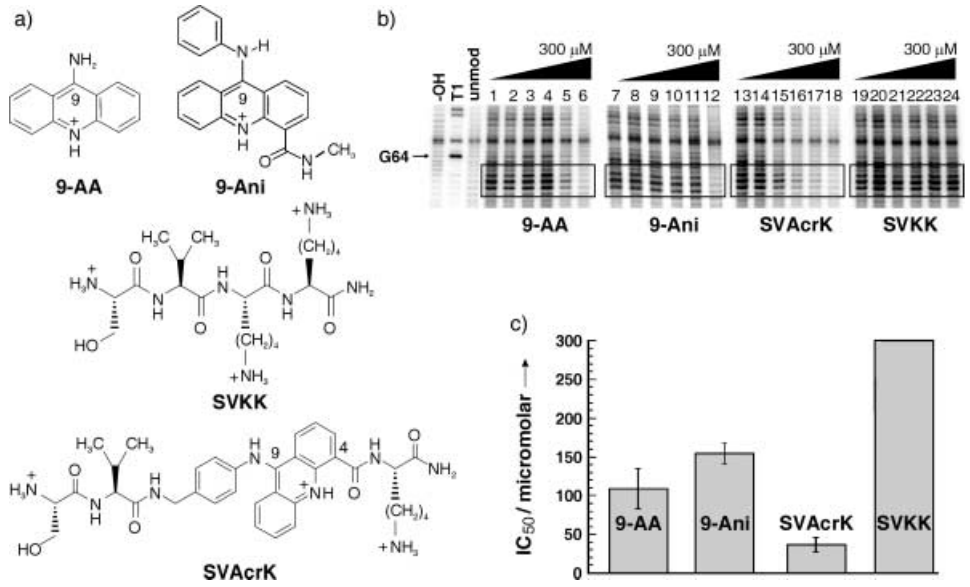


Figure 2. *a) Chemical structures of the small molecules used in preliminary screening. See the text for definitions. b) Affinity cleavage gel showing the major site (boxed) of PKR dsRBD cleavage of VA_I RNA.[42] For reaction conditions, see the Materials and Methods Section. Inhibition reactions were performed with increasing concentrations (0, 10, 20, 50, 100, 300 μM) of 9-AA (lanes 1 – 6), 9-Ani (lanes 7 – 12), SVAcrK (lanes 13 – 18), and SVKK (lanes 19 – 24). A control reaction was also carried out with unmodified E29C PKR dsRBD under identical conditions (lane "unmod"). c) Histogram plot of the IC_{50} values determined for each compound (average of three experiments). IC_{50} values: 9-AA, 109 ± 25 μM; 9-Ani, 155 ± 13 μM; SVAcrK, 37 ± 9 μM; SVKK, determined to be greater than the highest concentration tested (300 μM). Lane T1 = ribonuclease; Lane OH = alkaline hydrolysis.*

the requirement for acridine in the peptide sequence but maintain the same overall charge as SVAcrK.

Each of these compounds was assayed for its ability to prevent complex formation by titration of the small molecule into the affinity cleavage reaction between EDTA·Fe modified PKR dsRBD and VA_I RNA (see the Materials and Methods Section). It was previously shown that the intensity of the bands on a gel that results from an affinity cleavage reaction correlates with the fraction of nucleic-acid-binding molecule bound to its target.[45] We used this relationship to determine the relative potencies of inhibition by measuring differences in cleavage efficiency at the apical stem loop of VA_I RNA. In addition, these data were used to calculate an IC_{50} value for inhibition.

The differences in the relative potencies of the four compounds assayed are evident from the gel in Figure 2b. The control peptide with no acridine (SVKK) did not exhibit any inhibition, even at the highest concentration used ($IC_{50} >$ 300 μM). 9-AA and 9-Ani inhibited the affinity cleaving reaction with IC_{50} values of 109 ± 25 and 155 ± 1 μM, respectively. SVAcrK displayed an IC_{50} value of 37 ± 9 μM (Figure 2c), perhaps as a result of the presence of two additional positive charges with respect to the other compounds. These results indicate that a simple charged tetrapeptide cannot effectively prevent the binding of the PKR RBD to VA_I RNA. Interestingly, aniline substitution at the 9-position and simple carboxamide substitution at the 4-position had only a minimal effect on inhibition by the acridine intercalator. The variation seen may be caused by the aniline substituent, which lowers the basicity of the acridine ring nitrogen and thus the fraction of protonated species at pH 7. Each of the acridine intercalators blocked binding at higher concentrations.

The PAC is the most potent inhibitor of the four compounds tested in this initial screen. We conclude that both the peptide and acridine domains are required to achieve the observed potency of SVAcrK. An increase in the amount of positive charge on the molecule may in fact enhance the binding affinity for RNA, but at the price of specificity and limitation of access to the whole cell environment (that is, ability to permeate the cell). Therefore, we wished to define structural modifications of the PAC that would increase the potency of inhibition without increasing the overall charge of the molecule. For this reason, we prepared a combinatorial library of PACs that contained only neutral and uncommon amino acid monomers.

Library synthesis

A 108-compound library was synthesized by split-and-pool chemistry as nine mixtures of twelve PACs by using neutral and uncommon amino acids. Each compound in the library was synthesized by SPPS, capped at the N terminus with 9-anilinoacridine,[40] and has the general structure denoted as Acr-**X-Y-Z** (Figure 3a). The peptide substituents have an effect on the potency of the compound so we chose to initially optimize the peptide structure at the 4-position of the acridine ring for the purposes of this study.

The relatively small size of the library we chose to analyze initially (one that did not include any of the 20 common amino acids) led us to select monomers with unique functional group diversity to target the different structures found in RNA. Six different amino acids were used: D-asparagine (D-Asn, **a**), β-alanine (β-Ala, **b**), and *trans*-4-hydroxyproline (Hyp, **c**), 2-furylalanine (Fur, **d**), 4-nitrophenylalanine (NO_2F, **e**), and 1-napthyl-

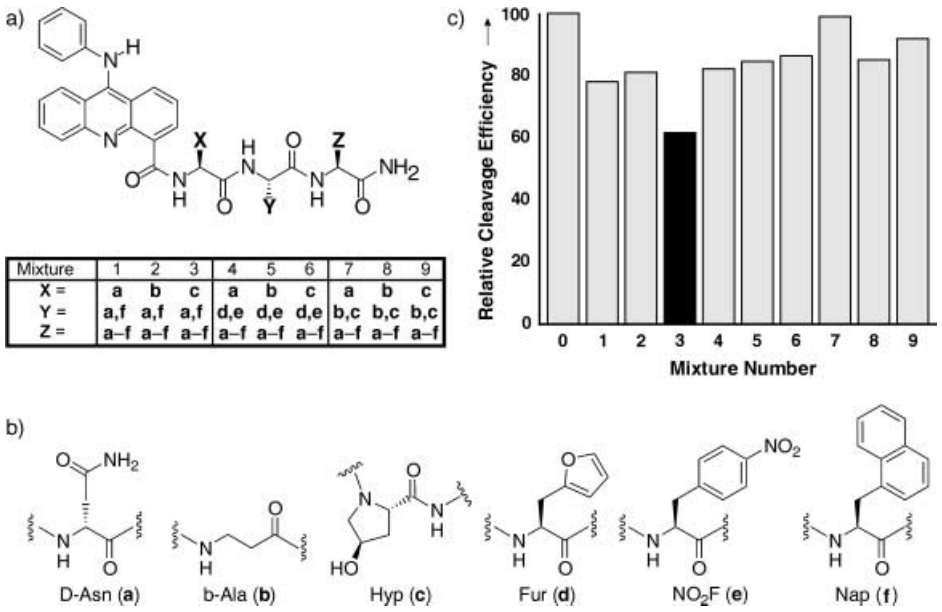
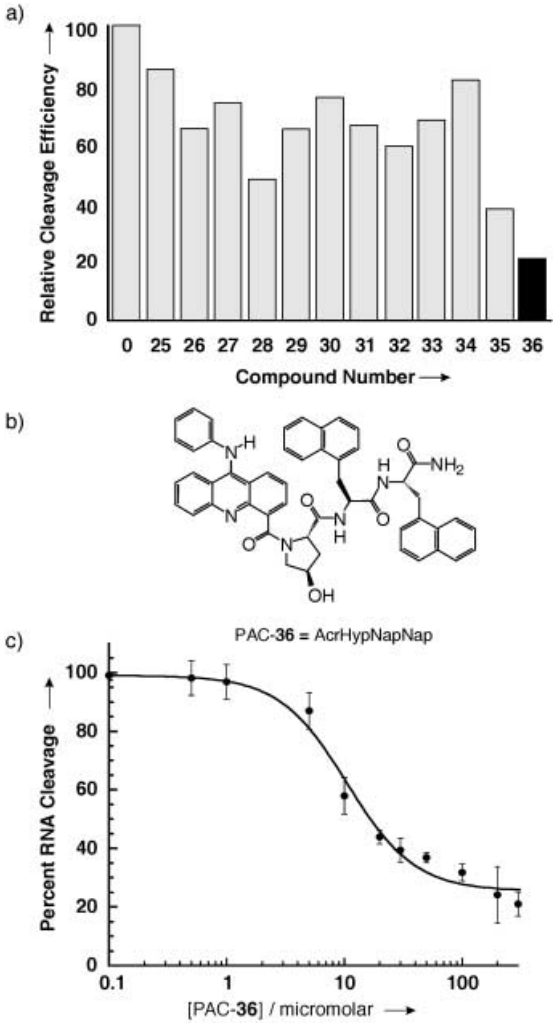


Figure 3. *a) Structure of the 108-member deconvolution library of peptide – acridine conjugates. b) Six neutral and uncommon amino acids were incorporated as groups* ***X***, ***Y***, *and* ***Z*** *of the peptide appended to the 4-position of the 9-anilinoacridine ring. The library was composed of 9 mixtures that contained 12 compounds each. c) Histogram plot of the relative cleavage efficiency for each mixture tested (average of two experiments). This value represents the potency of inhibition of the affinity cleaving reaction and is obtained as a ratio of the cleavage efficiency in the presence and absence of a fixed concentration of mixture (10 μM). Mixture 3 was determined to be the most potent, with the lowest relative cleavage efficiency.*



alanine (Nap, **f**; Figure 3b). We chose these amino acids to probe various structural parameters of the tripeptide, such as hydrogen bond donors/acceptors, flexibility, rigidity, and π-stacking.

Three of the nine mixtures of twelve compounds contained D-Asn as the **X** group, the next three had β-Ala, and the remaining three featured Hyp. The **Y** group consisted of one of three pairs of monomers: D-Asn and Nap, β-Ala and Hyp, or Fur and NO_2F. The **Z** group incorporated any one of the six possible amino acids mentioned. All mixtures were analyzed by using reversed-phase HPLC and electrospray ionization (ESI) mass spectrometry.[40] 94% of the expected library members were identified with few contaminating peaks in the mass spectra, which suggests a good level of purity of the mixtures for use in library screening.

Screening of the library and deconvolution

Each of the nine mixtures described above was tested for its ability to inhibit the binding of the PKR dsRBD to VA_I RNA. Each mixture was titrated into the affinity cleavage reaction in the same fashion as described earlier. Again, the relative cleavage efficiencies were compared and one mixture displayed significant inhibition (mixture 3, Figure 3c).

Figure 4. *a) Histogram plot of the relative cleavage efficiency of each individual compound from mixture 3. The amount of cleavage observed with no compound added is set to a value of 100% and is represented in column 0. The columns correspond to the PACs: Acr-Hyp-(D)Asn-(D)Asn (****25****), (D)Asn-βAla (****26****), (D)Asn-Hyp (****27****), (D)Asn-Fur (****28****), (D)Asn-NO_2F (****29****), (D)Asn-Nap (****30****), Nap-(D)Asn (****31****), Nap-βAla (****32****), Nap-Hyp (****33****), Nap-Fur (****34****), Nap-NO_2F (****35****), Nap-Nap (****36****). b) Chemical structure of PAC-****36****, the compound that displays the highest potency (lowest IC_{50} value) in the PKR affinity cleavage screening assay. c) Curve plotting the IC_{50} value for PAC-****36****. This value was determined to be 10 ± 5 μM (average of at least three experiments at each concentration).*

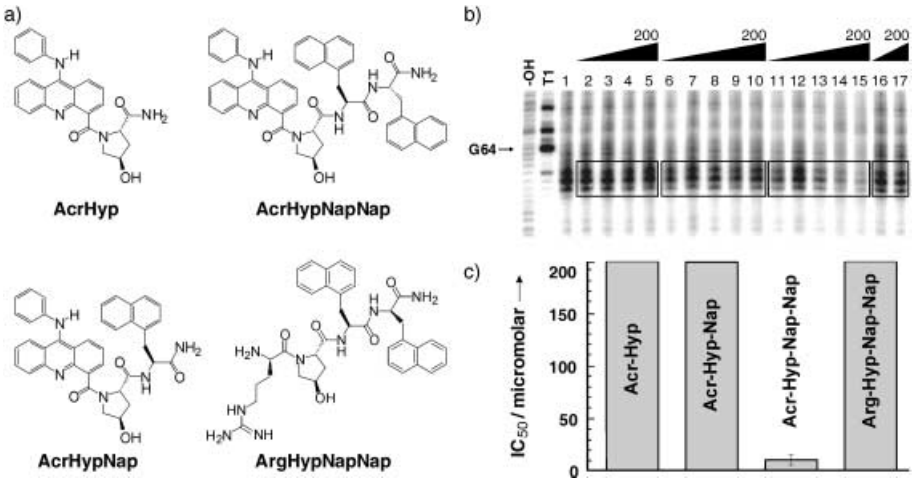


Figure 5. *a) Chemical structures of the compounds used in the structure – function analysis of PAC-**36**. b) Affinity cleavage gel showing the major site (boxed) of PKR dsRBD cleavage of VA_I RNA (for reaction details, see the Materials and Methods section). The inhibtion reactions were performed with no test compound added (lane 1), Acr-Hyp (lanes 2 – 5: 10, 30, 100, 200 μM), Acr-Hyp-Nap (lanes 6 – 10: 10, 30, 100, 200 μM), PAC-**36** (lanes 11 – 15: 0.1, 1, 10, 100, 200 μM), and Arg-Hyp-Nap-Nap (lanes 16 and 17: 100, 200 μM). c) Histogram plot of the IC_{50} values determined for each compound (average of three experiments). PAC-**36** displayed an IC_{50} value of 10 ± 5 μM, whereas Acr-Hyp, Acr-Hyp-Nap, and Arg-Hyp-Nap-Nap did not inhibit the reaction even at the highest concentration tested (200 μM).*

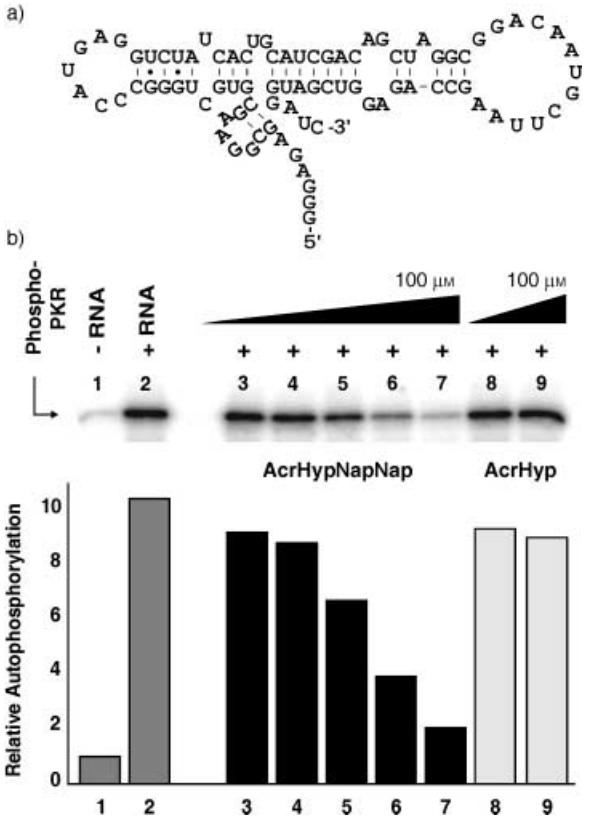


Figure 6. *a) Secondary structure of the 92-nt RNA aptamer used to activate full-length PKR in the autophosphorylation assay.[47] b) 15 % SDS gel and histogram of the results from a PKR autophosphorylation assay. Reactions were performed with full-length enzyme (~ 100 nM) and γ-^{32}P-ATP in the absence (no activation, lane 1) or presence (activation, lanes 2 – 9) of RNA aptamer (1.5 μM) in the reaction buffer. Reactions were carried out without a test compound (lanes 1 and 2), with PAC-**36** (lanes 3 – 7: 1, 5, 10, 50, 100 μM), and with Acr-Hyp (lanes 8 and 9: 1, 100 μM). The relative activation of PKR (phospho – PKR) was calculated as the ratio of autophosphorylation with and without the addition of test compound (average of two experiments).*

The peptide structures in mixture 3 had Hyp as the **X** group, either D-Asn or Nap as **Y**, and any one of the six amino acids as **Z**. Based on the screening profile of this mixture, all 12 members (compounds **25** – **36**; compounds **1** – **24** are those in mixtures 1 and 2) were resynthesized, HPLC-purified, and assayed individually. The results of deconvolution are presented in Figure 4a. PAC-**36** (Figure 4b) repeatedly displayed the best potency (lowest IC_{50} value) in the affinity cleaving assay, with an IC_{50} value of 10 ± 5 μM (Figure 4c). We were thus able to use the affinity cleavage assay to screen a library of PACs and select a compound with a charge-neutral substituent fused at the acridine 4-position that is more potent than SVAcrK (IC_{50} = 37 μM) and similar in potency to the highly positively charged aminoglycoside antibiotic, neomycin (7.5 μM). Analysis of neomycin inhibition of PKR dsRBD binding to VA_I RNA is shown in the Supporting Information.

Structure – function analysis of PAC-36

Next, we sought to define the minimum structural requirements for the most potent inhibitor, PAC-**36**. We synthesized the compounds Acr-Hyp, Acr-Hyp-Nap, and Arg-Hyp-Nap-Nap (Figure 5a) by SPPS. Acr-Hyp and Acr-Hyp-Nap are PAC derivatives with a truncated peptide substituent, and Arg-Hyp-Nap-Nap was prepared to verify the importance of 9-anilinoacridine in the sequence. Surprisingly, none of the three additional compounds prepared exhibited any potency for inhibition for PKR-dsRBD binding to VA_I RNA (up to 200 μM; Figure 5b and 5c). We conclude that both the acridine domain and the complete peptide sequence are responsible for the observed potency of PAC-**36**.

Testing the selectivity of PAC-36

Affinity cleaving studies have shown that the dsRBM I of PKR has a major binding site in the apical stem of VA_I RNA as well as a

minor site in the central domain.[42] PAC-**36** inhibits the binding at both sites with similar potency (see the Supporting Information). This result suggests that this compound does not have a high selectivity for the apical stem site on VA_I RNA and may inhibit the binding of PKR to other RNA ligands.

The binding of PKR to both kinase-activating and kinase-inhibiting RNA ligands was previously analyzed in our laboratory.[46] Consequently, we tested the selectivity of PAC-**36** for other RNA ligands of PKR. We assessed its ability to inhibit the affinity cleavage reaction with a 92-nucleotide (92-nt) activating RNA aptamer selected from a random pool of RNAs (Figure 6a).[47] PAC-**36** prevented complex formation between PKR and this RNA aptamer with a similar potency to that seen for VA_I RNA (data not shown), as was expected from the PAC-**36** inhibition of binding to both VA_I RNA binding sites. However, it is important to consider that all of these binding sites contain extensive duplex structure to which both PAC-**36** and the PKR dsRBD may bind.

The fact that the RNA aptamer is an activator of PKR kinase activity enabled us to test the inhibition potency of our PACs in an independent assay and confirm the structure–function relationships observed. When bound to an activating dsRNA and adenosine triphosphate (ATP), full-length PKR undergoes autophosphorylation reactions at multiple serine and threonine residues, which enables it to phosphorylate protein substrates such as histone IIA or eIF-2α.[10, 48] Figure 6b shows that PAC-**36** blocks the autophosphorylation of full-length PKR with an IC_{50} value of $40 \pm 7\ \mu M$. Acr-Hyp and Acr-Hyp-Nap display no observed inhibition (up to 100 μM), as predicted by affinity cleavage results.

Conclusion

The results reported here demonstrate that a screening assay based on the affinity cleavage of RNA ligands by an EDTA·Fe modified mutant of the PKR dsRBD can effectively assess the ability of individual molecules, as well as libraries of compounds, to inhibit the formation of specific PKR–RNA complexes. A low-micromolar inhibitor of PKR–RNA binding was discovered by using this approach. This assay has the benefit of not only testing the potency of the inhibitor, but also the selectivity if multiple binding sites exist on the RNA target. Although PAC-**36** is not selective for a specific site on VA_I RNA, it is clear that the complete structure of the peptide appendage controls its potency. Furthermore, structural requirements for inhibition of PKR–RNA binding by the selected peptide–acridine conjugate were substantiated in an independent PKR autophosphorylation assay.

All of the PACs found in mixture 3 had limited water solubility and we hypothesize that this characteristic contributes to the ability of PAC-**36** to inhibit PKR–RNA binding (calculated partition coefficient, Clog$P = 8.0$). This solubility could also explain why the truncated derivatives did not compare well. In solution, PAC-**36** might bury hydrophobic side chains into pockets presented by the protein–RNA complex, perhaps between base pairs in the duplex secondary structure. Naphthalene has been shown to stack in a duplex structure more strongly than any of the natural bases.[49] Additional experiments are required to fully define the mechanism of inhibition by PAC-**36**.

Materials and Methods

Library and single compound synthesis: Each PAC library (mixtures 1–9), individual PAC (SVAcrK, PACs **25**–**36**, Acr-Hyp, Acr-Hyp-Nap) and peptide (SVKK, Arg-Hyp-Nap-Nap) was synthesized by using 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids (Advanced ChemTech & PepTech) on Rink Amide resin (NovaBiochem; 0.72 mmole g^{-1} loading) according to standard SPPS protocols.[50–51] Use of 9-anilinoacridine acids has been previously described.[40–41] Cleavage from the resin was accomplished with a solution of trifluoroacetic acid (TFA)/triisopropylsilane/H_2O (95:2.5:2.5). The solution was separated between water and diethyl ether and then the aqueous layer was neutralized with triethylamine and concentrated by lyophilization. PAC libraries were loaded onto a Sep-Pak cartridge (Waters), eluted with an 80:20 mixture of CH_3CN/H_2O in TFA (0.1%), concentrated, and characterized by reversed-phase HPLC and ESI mass spectrometry. Individual PACs and peptides were HPLC-purified on a reversed-phase C18 column (4.6 × 250 mm, Vydac) over 60 min with a flow rate of 2 mL min^{-1}. Stock solutions of each library mixture and of individual compounds were prepared to uniform UV absorbance ($\lambda = 442$ nm) assuming an average extinction coeffient of 4000 $M^{-1}cm^{-1}$.

RNA preparation: VA_I RNA was generated by transcription with T7 RNA polymerase as previously described,[42] dephosphorylated with shrimp alkaline phosphatase (SAP; Pharmacia) and purified on a 12% denaturing polyacrylamide gel. The 92-nt RNA aptamer was also generated by transcription with T7 RNA polymerase as previously described,[47] dephosphorylated with SAP, and purified on a 16% denaturing polyacrylamide gel. Dephosphorylated RNAs were 5′-^{32}P end-labeled with [γ-^{32}P]-ATP and again gel purified by following standard protocols.[52]

Affinity cleaving screening assay: The RBD of PKR was mutated (E29C) and modified with EDTA·Fe as previously described.[42] Protein–RNA complexes were formed by mixing PKR RBD (1 μM) with 5′-end-labeled VA_I RNA at RT for 7 min in tris(hydroxymethyl)aminomethane (Tris)–HCl (25 mM; pH = 7.0), NaCl (10 mM), and tRNA (30 $\mu g\ ml^{-}$). Although this concentration of tRNA may affect the relative IC_{50} values for the compounds tested, a rigorous investigation was not performed. It is possible that our ligands bind tRNA as well, but it is also known that the presence of a carrier nucleic acid affects the dissociation constant of PKR for dsRNA.[53] PKR-RBD–RNA complexes (20 μL final reaction volume) were further incubated with increasing concentrations of test compound (in 6% methanol) for 5 min at RT. These mixtures were probed by initiation of hydroxyl radical formation with H_2O_2 (0.01%) and sodium ascorbate (5 mM) at RT for 3 min. Reactions were quenched with water (80 μL) followed by phenol/chloroform extraction and ethanol precipitation. Cleaved RNA was resuspended in loading dye (7 μL; 96% formamide in 0.2X Tris-borate EDTA with xylene cyanol dye) and analyzed on a 12% denaturing polyacrylamide gel. Data were obtained from the gels by using storage phosphor autoradiography and a STORM phosphorimager (Molecular Dynamics). Analysis of the cleavage data was performed with Image Quant software (Molecular Dynamics).

PAC inhibition of PKR autophosphorylation: Full-length PKR was expressed and purified as previously described.[35] Autophosphorylation reactions were performed as follows: PKR (100 nM approximate final concentration) was added to a sample with or without the

92-nt RNA aptamer (1.5 μM) in a Tris – HCl (20 mM, pH = 7.6) buffer that contained KCl (100 mM), EDTA (0.1 mM), and $MgCl_2$ (2 mM) and kept on ice for 10 min. PKR – RNA complexes were then incubated with increasing concentrations of PACs for 5 min at RT. Histone IIA and [γ-^{32}P]ATP were then added to final concentrations of 250 μg mL^{-1} and 2 μM, respectively, and kinase reactions were allowed to proceed for 5 min at 30 °C. The reactions were placed on ice and subsequently quenched by boiling in SDS-PAGE loading buffer. The products were resolved by 15% SDS-PAGE. Labeled proteins were visualized and band intensities were quantified by storage phosphor autoradiography as above.

IC_{50} curve calculations: The cleaved nucleotides 58, 59, and 60 of VA_I RNA were quantified and combined for each compound concentration (see Figures 2b and 5b). The unmodified protein lane was used as the background and the 0 μM compound lane was fixed at 100% RNA cleavage. All other lanes were calculated as % cleavage, which signifies the amount of RNA bound by protein. An IC_{50} for each compound was calculated by fitting all data to the equation,

$$\%\ \text{cleavage} = (\text{range}/1 + ([\text{PAC}]/IC_{50})^{\text{slope}})) + \text{background}$$

with the least squares method of the KaleidaGraph software. Each experiment was carried out in triplicate and plotted values are averages ± standard deviation.

Calculated log*P* (Clog*P*): The calculated log*P* value for PAC-**36** was obtained by using the ChemOffice Ultra 2001 software (CambridgeSoft) and the ChemSAR plug-in for the Excel 2000 program (Microsoft). This parameter is a measure of the lipophilicity of a compound. The Clog*P* value obtained for PAC-**36** is 8.0. For comparison, the Clog*P* value for the antitumor drug amsacrine is 4.7.

Supporting information: HPLC and mass spectral data for mixture 3 and individual PACs and complete VA_I RNA affinity cleaving gels for 9-AA, 9-Ani, SVAcrK, SVKK, mixture 3, PAC-**36** (Acr-Hyp-Nap-Nap), and neomycin are available in the Supporting Information.

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