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Modulation of the Rev–RRE Interaction by Aromatic Heterocyclic Compounds

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Abstract—The HIV-1 Rev protein regulates the nucleocytoplasmic distribution of viral precursor RNAs that encode HIV-1 structural proteins. Rev-mediated viral RNA expression requires a sequence-specific interaction between Rev and a viral RNA sequence, the Rev responsive element (RRE). Because the Rev–RRE interaction is essential for HIV-1 replication, anti-viral agents that selectively block this interaction may be effective anti-HIV-1 therapeutics. Here, we show that certain aromatic heterocyclic compounds, in particular, a tetracationic diphenylfuran, AK.A, can block binding of Rev to its high-affinity viral RNA binding site. AK.A abolishes Rev–RRE interactions at concentrations as low as 0.1 μM. Inhibition appears to be selective and results from competitive binding of the drug to a discrete region within the Rev binding site. Interestingly, the molecular basis for the AK.A–RNA interaction, as well as the mode of RNA binding differs from previously described aminoglycoside Rev inhibitors. Analysis of a variety of aromatic heterocyclic compounds and their derivatives reveals stereo-specific features required for the inhibition. Our results further demonstrate the feasibility of identifying and designing small molecules that selectively block viral RNA–protein interactions. © 1997 Elsevier Science Ltd.

Introduction

The genome of human immunodeficiency virus type 1 (HIV-1) contains three major coding regions *gag*, *pol*, and *env*, and long terminal repeats regulatory sequences (LTRs) common to known retroviruses. The viral open reading frames that have been mapped within these three regions encode the viral structural proteins, enzymes, and surface glycoproteins.[1–5] Additionally, novel HIV-1 regulatory and accessory proteins are encoded by viral sequences located in subregions of the HIV-1 genome.[2,4,5] These additional HIV-1-encoded proteins appear to function at different stages of the viral life cycle.[6–15] Clinical efforts to block HIV-1 replication have focused on inactivation of the viral reverse transcriptase (RT), and more recently, the viral protease.[16,17] It is unclear whether current anti-HIV agents will retain potent inhibitory activity over time, in the absence of any adverse effects. Thus, the need for efficacious anti-HIV-1 therapeutics remains a focus of academic and industrial research pursuits. However, these efforts are no longer directed at developing anti-viral agents that inactivate the HIV-1 enzymes, but rather, at designing inhibitors that target essential viral RNA–protein interactions.[18–28] Such anti-HIV agents could inhibit a specific viral RNA–protein interaction by binding directly to the viral mRNA or an essential viral protein.

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Rev as a target

Rev, an HIV-1 regulatory protein, acts post-transcriptionally to selectively increase the cytoplasmic levels of *gag*, *pol* and *env* mRNAs.[2–4,29–31] Rev contains an arginine-rich binding domain which interacts with a *cis*-acting RNA element in the viral RNA designated the Rev responsive element (RRE) and a discrete carboxy-terminal region or 'effector domain' which interacts with cellular factors.[30,32–37] Because Rev is essential for HIV-l replication, it is an attractive target for therapeutic intervention.[16,17] Several novel strategies have been described to reduce Rev protein or block its mechanism of action.[18–22,24,25,27,28] In each case, the anti-Rev agent is an oligonucleotide or protein, rather than a conventional small organic molecule. The large size of these Rev inhibitors makes their intracellular delivery, the site of Rev function, more difficult.[17]

Small molecule inhibitors of Rev function

We have previously reported that the aminoglycosides neomycin B and tobramycin selectively inhibit Rev binding in vitro and block virus production in vivo.[26] Neomycin B and tobramycin interact directly with a

noncanonical base pair that is essential for Rev recognition and binding.[33] Although neomycin B and tobramycin are specific inhibitors of the Rev–RRE interaction, their low cellular permeabilities and toxicities, characteristic of aminoglycosides, limit their use as anti-Rev therapeutics.[38–41] The initial rationale to test whether aminoglycosides blocked Rev binding was based on previous studies that indicated aminoglycosides could bind directly to ribosomal RNA.[42–51] However, these reports of RNA–aminoglycoside interactions were preceded by extensive analyses of small molecules that bound in the major or minor grooves of DNA or intercalated into DNA helices.[52,53] These studies demonstrated that a variety of small molecules could interact directly with DNA by different binding modes. Recent biophysical studies of nucleic acid–small molecule interactions have indicated that RNA, as well as DNA, can bind a variety of small molecules with different binding mechanisms.[53–59] Taken together, these results suggested the feasibility of identifying other, non-aminoglycoside small molecules that selectively inhibit the Rev–RRE interaction and ultimately, HIV-1 replication. To address this possibility, we tested a series of related aromatic heterocyclic compounds (aromatic heterocycles), particularly diphenylfuran derivatives which have been shown to bind to RNA in a structure-dependent manner.[59]

Here, we describe the identification of certain aromatic heterocycles that specifically inhibit the Rev–RRE interaction. The molecular basis of the inhibition involves a direct interaction between the small molecule and the RNA. Structure–activity profiles of the diphenylfurans and related aromatic heterocycles indicate that potent inhibitory activity requires cationic alkylamine substituents and a central aromatic ring.

Results

To test whether diphenylfuran derivatives block binding of HIV-1 Rev to the RRE, *Escherichia coli*-derived Rev was incubated with a ^{32}P-labeled 94.7 RNA probe containing the high-affinity Rev-binding site in the presence of various aromatic heterocycles.[26] Binding of Rev to the 94.7 RNA probe was quantitated by an RNA gel mobility shift assay.

We initially assayed 17 diphenyl and three trisubstituted furans, five diphenyl and two trisubstituted pyrroles, and three disubstituted oxazoles for their ability to inhibit Rev binding. These 30 heterocycles had been previously assayed for their ability to bind to model DNA duplexes (data not shown). All of the aromatic heterocycles tested contain two or more benzene substituents with alkylamine groups. The alkylamine substituents have either an open-chain or cyclic configuration. Some of these data are shown in Figure 1. The structure of each compound tested and the micromolar concentration (μM) that inhibits 50% of the Rev–RRE interaction (IC_{50}) are presented in Table 1. From these results, we conclude that several of these aromatic heterocyclic compounds have significant inhibitory potential. In particular, the tetracationic diphenylfuran, AK.A was a potent inhibitor of Rev binding at concentrations as low as 0.1 μM. AK.A inhibits the Rev–RRE interaction at a 10-fold-lower concentration than neomycin B, an aminoglycoside antibiotic previously shown to selectively inhibit the Rev–RRE interaction.[26] Two additional diphenylfurans, furandiamidine and furimidazole, which both lack the R-substituents of AK.A were inhibitory only at concentrations greater than 1 mM. The remaining cationic diphenylfuran derivatives, AK.48s, AK.54, AK.55, AK.58s AK.62, AK.71s, AK.154, AK.167, and AK.175 differ significantly in their ability to block Rev binding. Two AK.A derivatives, AK.181 and AK.191 had no Rev inhibitory activity at any concentration tested.

Inhibitory activity of AK.A derivatives

To identify the chemical moieties of AK.A that are required for inhibition, we analyzed several structurally related aromatic heterocycles in an in vitro Rev binding assay (Table 1). Such aromatic heterocyclic compounds included trisubstituted furans (AK.67s and AK.72s), substituted diphenylpyrroles (AK.18, AK.132), tetrasubstituted pyrroles (AK117, AK.118), and substituted diphenyloxazoles (AK.26 and AK.107). Of these compounds, AK.18 inhibited Rev binding at a concentration similar to that of furimidazole, while AK.132 and AK.117 were less effective than furandiamidine. We also tested pentamidine, an open-chain heterocycle containing the same alkylamine R-group substituents as furandiamidine, but with a diphenoxypentane in place of a substituted furan ring. Pentamidine can serve as a parent heterocycle for the synthesis of furandiamidine by undergoing ring closure. We found pentamidine to be a very poor inhibitor of Rev binding even at concentrations greater than 100 μM. These results suggest that alkylamine substituents are essential, but not sufficient, for inhibition. The presence of a central resonance-stabilized heterocycle, in this case, a five-membered aromatic ring, significantly enhances inhibition.

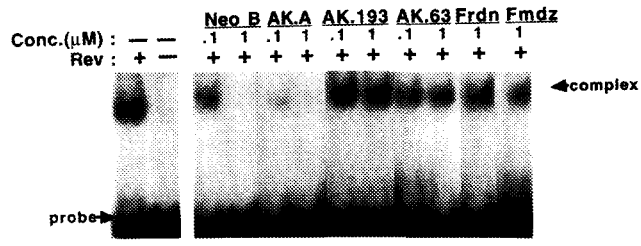
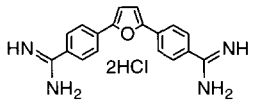
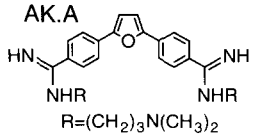


Figure 1. Diphenylfuran derivatives inhibit binding of the HIV-1 Rev to the RRE. A 67 nt ^{32}P-labeled RNA probe containing a high-affinity HIV-1 Rev binding site was incubated with purified *E. coli*-derived HIV-1 Rev protein in the absence or presence of various aromatic heterocyclic compounds.[26] The compound tested and its concentration (Conc.) (in μM) are indicated above each lane. A control reaction in the absence of drug is shown at the left (−). The absence (−) or presence (+) of HIV-1 Rev is also indicated. The HIV-1 Rev–RRE complex and the unbound probe are indicated by arrows. Binding reactions were performed as previously described.[26]

Table 1. Structures and activities of diphenylfuran derivatives. Compounds are arranged based on their R-group substituents relevant to diphenylfurandiamidine and AK.A. The inhibitory concentration (in μM) for >50% reduction in Rev binding (IC_{50}) is indicated to the right of each compound. Concentration error values for these compounds were estimated at ± 0.005–0.02 μM. The structure and activity profiles for pentamidine and other aromatic heterocyles described in the text are included. For visual simplicity, the positive charges on the amide substituents have been omitted

Furandiamidine	>1
	
AK.A	<1
	
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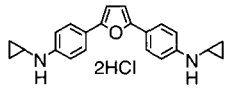	
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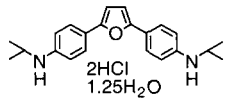	
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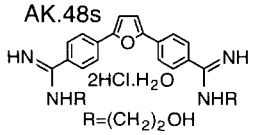
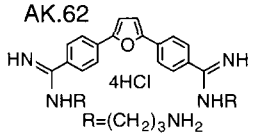
	
AK.62	10–100
	
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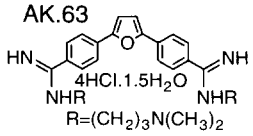	
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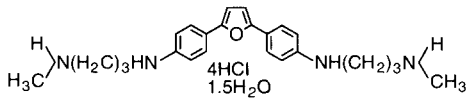	
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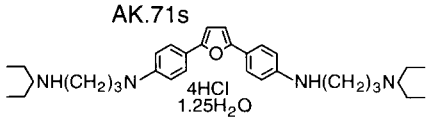	
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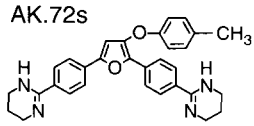	
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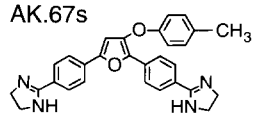	
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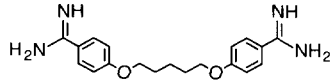	
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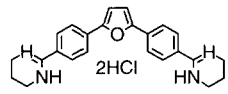	
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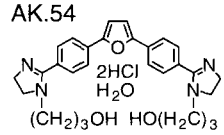	
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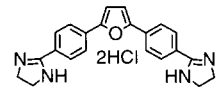	
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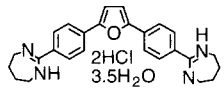	
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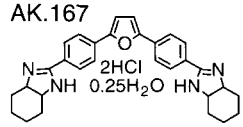	
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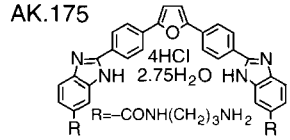	
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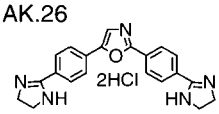	
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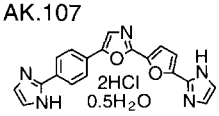	
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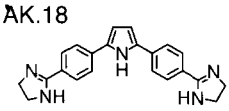	
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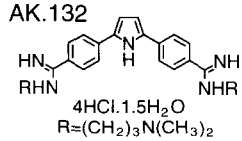	
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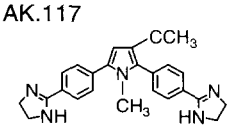	

Diphenylfuran derivatives do not inhibit mammalian pre-mRNA splicing

To test whether inhibition was specific for the Rev–RRE interaction, we examined the effect of the inhibitory diphenylfurans on in vitro pre-mRNA splicing. Because pre-mRNA splicing involves numerous RNA–protein and RNA–RNA interactions, it serves as a stringent, biologically relevant assay for specificity.[61,62] We have previously used this assay to demonstrate that the aminoglycosides neomycin B and tobramycin are selective inhibitors of the Rev–RRE interaction.[26] Figure 2 shows the results from in vitro splicing reactions containing 50 μM neomycin B, AK.A, or furandiamidine. At concentrations that are 50- to 500-fold higher than that required to abolish Rev binding, neither of the diphenylfuran derivatives affected splicing of an adenovirus major late pre-mRNA substrate in a HeLa cell nuclear extract. In addition, 200 μM AK.A does not inhibit the HIV-1 TAT–TAR or HTLV-I Rex–XRE interactions (data not show). Taken together, these data indicate that the inhibition of the Rev–RRE interaction by diphenylfuran derivatives is specific.

AK.A binds to a discrete region of the RRE

Previous Rev inhibition studies, and more recent spectroscopic analysis of AK.A–nucleic acid interactions suggested that AK.A may inhibit Rev binding by direct interaction with the RRE.[59] To test this possibility, we performed chemical modification-footprinting experiments (Fig. 3). The 94.7 RNA was incubated with increasing concentrations of AK.A and treated with dimethylsulfate (DMS), 1-cyclohexyl-3-(2-morpholino-ethyl)-carbodiimide metho-*p*-toluene sulfonate (CMCT), or kethoxal. DMS methylates guanosine at N7 > adenosine at N1 > cytosine at N3; CMCT modifies at N3 of uridine > N1 of guanosine, and kethoxal modifies at N1 and N2 of guanosine.[26,44,45] Modification of specific bases was detected by primer extension.

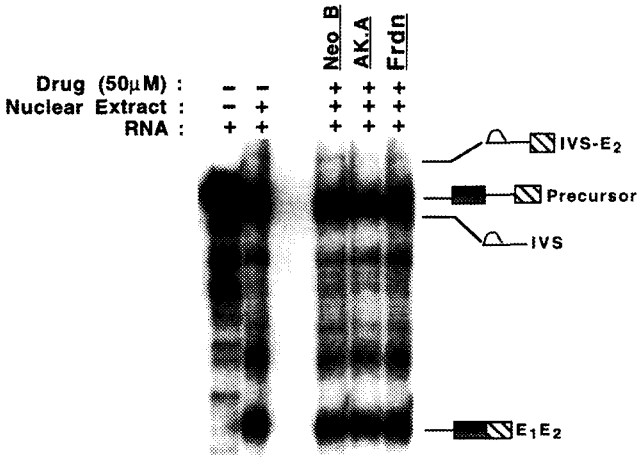

Figure 2. The inhibitory diphenylfurans do not affect pre-mRNA splicing. A ^{32}P-labeled adenovirus major late pre-mRNA (MVSXL) was incubated in a HeLa nuclear splicing extract in the absence (−) or presence (+) of 50 mM neomycin B, AK.A, or furandiamidine.[26] The structure of each reaction product is indicated at the right. The control splicing reaction in the absence (−) of drug is shown at the left.

The cleavage pattern of unbound 94.7 RNA treated with DMS and kethoxal is similar to that previously reported (Fig. 3, lane K).[26,63] The different chemical reactivities of DMS, CMCT, and kethoxal with the stem IIA nucleotides is most likely explained by differential access to this double-stranded region of the RRE. Nucleotides protected from chemical modification by AK.A are located primarily outside the core binding element, within stem IIA of the RRE. Specifically, nucleotides A44, U99, and U102 are the most strongly protected by AK.A. These nucleotides are not protected from chemical modification by 10 μM neomycin B or Rev protein. Similar concentrations of the non-inhibitory diphenylfuran AK.193 did not protect the RRE from chemical modification (data not shown). Binding of AK.A may sufficiently destabilize the A44–U99 and A42–A102 base pairs of the RRE, preventing Rev recognition and binding. In the presence of AK.A, we observed a strong protection of nucleotide C74, which is located at the most 3′-end of the core binding element. This nucleotide is also required for Rev recognition and binding and is strongly protected from chemical cleavage by the protein. Nucleotide C74, however, is not protected from chemical modification by 10 μM neomycin B. These data are summarized in Figure 3(B). AK.A can interact with nucleotide C74, disrupting the 46G:C74 base pair that is essential to core binding element formation and Rev binding. However, AK.A also interacts with nucleotides that are not protected by Rev or neomycin B.

Discussion

We have shown that certain aromatic heterocyclic compounds are potent and specific inhibitors of Rev binding. Our data indicates that the ability of an aromatic heterocycle to block an RNA–protein interaction is specific. Chemical footprinting data confirm that certain aromatic heterocycles, in particular the diphenylfuran tetracation, AK.A, interacts directly with specific nucleotides of the RRE, providing an explanation of this specificity. The nucleotides protected from modification by AK.A are located in two discrete regions of the RRE, specifically, within stem IIA and the core binding element. These two regions of the RRE are essential components of the core binding site secondary structure. The strong interaction between AK.A and stem IIA nucleotides most likely disrupts the Watson–Crick base-pairing characteristic of this double-stranded region of the RRE. Moreover, interaction between AK.A and nucleotide C74 would preclude formation of the C46:G74 base pair in the core Rev-binding element, thus preventing Rev binding to the RRE.

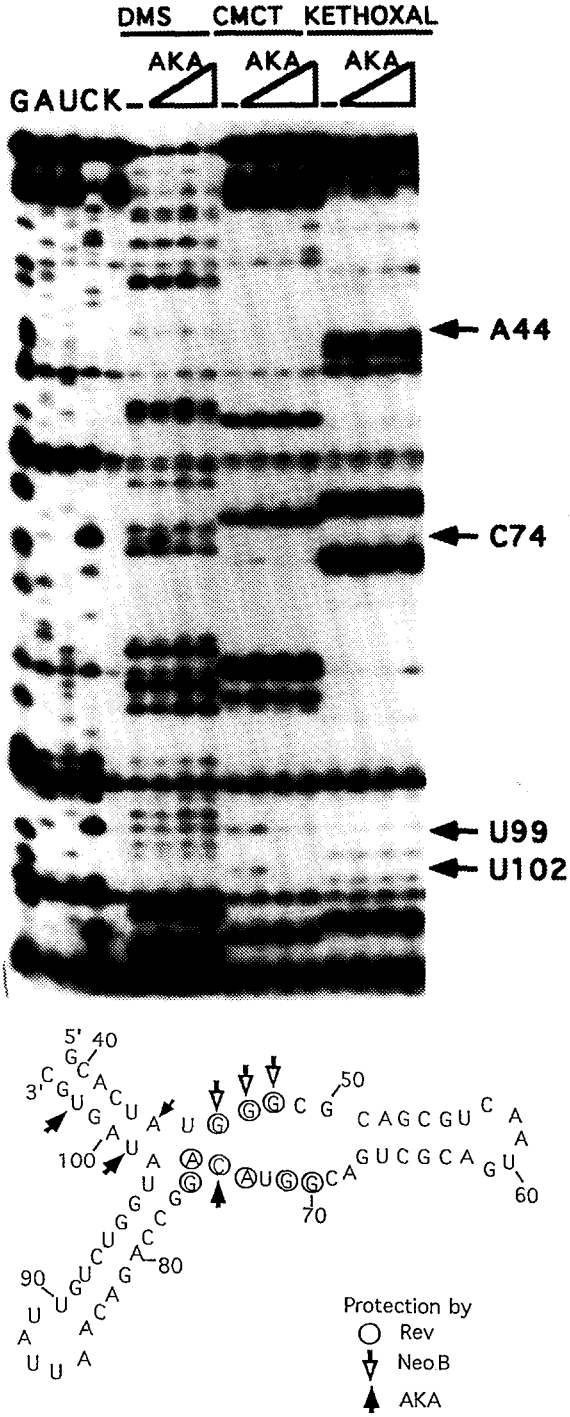

Figure 3. AK.A binds to a discrete region of the RRE. (A) DMS, CMCT, and Kethoxal modification/protection experiments. A 67 nt RNA probe (94.7) containing the high-affinity Rev binding site was incubated alone (−) or with increasing concentrations (0.1, 1, and 10 mM) of AK.A and treated with DMS, CMCT, or kethoxal as indicated.[26,44,45] The reverse-transcribed RNA, along with dideoxy-sequencing reactions (G, A, U, and C) and a control reaction with untreated 94.7 RNA (K) were resolved on a 8% denaturing polyacrylamide gel and visualized by autoradiography. Nucleotide positions discussed in the text are indicated to the right of the autoradiogram by arrows. (B) Summary of the chemical protection by AK.A, neomycin B, and Rev. Nucleotides strongly protected (>75%) from modification by AK.A are indicated by blue arrows; nucleotides strongly protected (>75%) from modification by neomycin B are indicated by red arrows, and nucleotides strongly protected (>75%) from modification by Rev are enclosed by a circle. Nucleotide assignments are as previously described.[19,26]

The molecular basis of AK.A inhibition is different from that of aminoglycosides

Our results suggest that the diphenyfuran tetracation, AK.A, specifically inhibits the Rev–RRE interaction in an RNA structure-dependent manner. We have previously demonstrated that nucleotide C74, which in concert with G46, forms the critical first Watson–Crick base pair of the core binding site, is also strongly protected from modification by the aminoglycoside Rev inhibitors neomycin B and tobramycin.[26] Thus, C74 and its base-pairing interaction with G46 appear to be important requirements for small-molecule inhibition of Rev binding. The requirement for small molecules to block the Rev–RRE interaction by selectively disrupting the G46:C76 base pair is consistent with previous observations that this base pair is essential for Rev function and strongly protected from chemical modification by Rev.[26,33,63] Thus, the molecular basis of the AK.A–RRE and aminoglycoside–RRE interactions are distinct.

Our chemical modification data are consistent with recent spectroscopic analysis of diphenylfuran derivatives binding to model poly(A)–poly(U) RNA duplexes and RRE hairpin substrates.[59] These studies have shown these compounds exhibit a wide range of RNA binding affinities and modes of interaction. In the case of AK.A, binding to the RRE occurs by a nonclassical intercalation and groove binding mechanism, which is distinct from the groove binding by neomycin B.

Structure–activity relationships

The structure–activity data in Table 1 indicate that aromatic heterocycles that are potent inhibitors of the Rev–RRE interaction contain alkylamine substituents, most often as an open-chain tertiary amine. The ability of AK.A and other aromatic heterocycles to inhibit the Rev–RRE interaction may also be affected by the spatial presentation of the alkylamine substituents, suggesting that a stereospecific orientation of these alkylamine groups is preferred for inhibition. Thus, alkylamine substituents that possess some degree of rotational freedom may be needed within the aromatic heterocycle structure to achieve the molecular conformations that block Rev binding.

The results of fluorescence titration experiments that monitored binding of AK.A to the RRE have indicated that on its binding to and intercalation in the RNA, the alkylamine substituents are located within the groove of A-form RNA.[59] The different Rev inhibitory activities observed with the cationic diphenylfurans and related aromatic heterocyclic compounds may be due to differences in the nucleophilic properties of their alkylamine substituents. The inhibitory activity of a given cationic diphenylfuran or related aromatic heterocycle may be directly related to its ability to form hydrogen-bond interactions with the RNA.

Aromaticity affects inhibition

The structure–activity relationships presented in Table 1 indicate that aromatic heterocyclic inhibitors of the Rev–RRE interaction contain either an oxygen or a nitrogen noncarbon constituent(s) in their central five-membered ring. Additionally, each compound has the resonance-stabilized *p* electron density (resonance energy) characteristic of aromatic molecules. The resonance energy component presented by an aromatic heterocycle modulates the overall stability of the heterocycle–RNA interaction formed, and therefore the Rev inhibitory activity of these heterocyclic compounds. Whether this resonance-energy mediated stability is accomplished by specific charge transfer reactions, π-orbital stacking, or an alternative mechanism is unclear. Depending on the electronegativity of the noncarbon element(s), the type of alkylamine substituents, as well as the number and composition of the central heterocyclic ring substituents can enhance its ability to block the Rev–RRE interaction.

The notion of the central aromatic heterocycle contributing to the inhibitory activity of these compounds is further supported by the inability of pentamidine, which has the same alkylamine substituents as furandiamide but an aliphatic chain in place of a heterocyclic ring, to inhibit the Rev–RRE interaction. In addition to having a different electron density, pentamidine may fail to inhibit Rev binding because its central pentane moiety hinders steric interactions between the alkylamine substituents and the RNA. Alternatively, the absence of structural preorganization within pentamidine may restrict its ability to selectively interact and block the Rev–RRE interaction.

Perspective

An increased understanding of aromatic heterocycle–RNA interactions should facilitate the design of small molecules that have even greater inhibitory activity and specificity for the Rev–RRE interaction. Using experimental approaches similar to those described here, we anticipate that we will be able to identify small-molecule inhibitors that selectively block other viral protein–nucleic acid interactions. Moreover, the current efforts by other groups to define the molecular and structural entities that mediate small molecule–RNA recognition will facilitate the future design of small-molecule therapeutics. We are currently testing additional aromatic heterocyclic compounds for their ability to inhibit the Rev–RRE interaction. The structure–activity profile derived from our initial studies of aromatic heterocycles will be useful for the future design of more potent aromatic heterocyclic inhibitors. The nonchiral nature of the inhibitory diphenylfurans and related aromatic heterocycles, as well as their ability to undergo electrophilic substitution reactions, make the rapid synthesis of additional aromatic heterocyclic derivatives more feasible.

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